THE NUTRITIONAL AND ANTHELMINTIC EFFECTS OF *CALLIANDRA CALOTHYRSUS* CONDENSED TANNIN IN THE GASTROINTESTINAL TRACT OF MERINO SHEEP

Thesis submitted

by

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STATEMENT OF THE CONTRIBUTION OF OTHERS

I, the author, wish to recognize that this thesis could not have been completed without the grateful assistance of the below mentioned individuals and organizations.

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DECLARATION OF ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *National Statement on Ethics Conduct in Research Involving Human* (1999), the *Joint NHMRC/AVCC Statement and Guidelines on Research Practice* (1997), the *James Cook University Policy on Experimentation Ethics. Standard Practices and Guidelines* (2001), *and the James Cook University Statement and Guidelines on Research Practice* (2001). The proposed research methodology received clearance from the James Cook University Ethics Review Committee (approval number A826_03).

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LIST OF ABBREVIATIONS

A	abomasum
ADD	Aminoacetonitrile derivative
AF	artificial fluid
ANOVA	analysis of variance
BAPNA	N-alpha-benzoyl-DL-arginine- p-nitroanilide
BZ	benzimidazole
Ca	caecum
Co	colon
CP	crude protein
BSA	bovine serum albumin
CT	condensed tannin
DEAE	diethylaminoethyl
DM	dry matter
DMD	dry matter digestibility
DMSO	dimethyl sulfoxide
D	duodenum
ECL	enterochromaffin-like
EHA	egg hatch assay
EN	ensheathed larvae
ES	excretory/secretory
EX	exsheathed larvae
F	faeces
FEC	faecal egg counts
GI	gastrointestinal
HCl	hydrochloric acid
HPLC	high pressure liquid chromatography
Hc	*Haemonchus contortus*
Hs	*Haemonchus contortus* susceptible
Hr	*Haemonchus contortus* resistant
I	ileum
IZ	imidazothiazole
L1	first stage larvae
L3	infective third stage larvae
L4	Fourth stage larvae
LWG	live weight gain
LDVA	larval development/viability assay
LEA	larval exsheathment assay
LFIA	larval feeding inhibition assay
MLs	macrocyclic lactones
$MgCl_2$	magnesium chloride
LMIA	larval migration inhibition assay
MANOVA	multivariate analysis of variance

MMC	migrating myoelectric complexes
N	nitrogen
ND	not detectable
NE	no effect
PEG	polyethylene glycol
PVPP	polyvinylpolypyrrolidone
R	rumen
RF	rumen fluid
SDS	sodium dodecyl sulfate
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tc	*Trichostrongylus colubriformis*
Ts	*Trichostrongylus colubriformis* susceptible

ABSTRACT

Calliandra calothyrsus is a high protein, high condensed tannin legume with potential nutritional value and possible anthelmintic properties. The objectives of these studies were to determine the *in vivo* and *in vitro* nutritional and anthelmintic properties of condensed tannin from this tropical shrub legume.

Comparisons were made between the methods of Terrill et al. (1992b) and Perez-Maldonado (1994) analysing the three fractions of condensed tannin (free, protein and fibre-bound) to determine which method is more suited for routine analysis of both plant material and digesta. Analysis of plant material for total condensed tannin was not different using either of the methods tested. However, when the three fractions of condensed tannin, free, protein-bound and fibre-bound, were analysed, then the modified method of Terrill et al. (1992b) gave a higher result for analysis of plant material. Total condensed tannin in rumen digesta when analysed with the method of Perez-Maldonado (1994) compared to the modified method of Terrill et al. (1992b) did result in a significantly higher value, however, results for the different fractions of condensed tannin were not significantly different.

The *in vivo* experiment compared digesta from lambs fed high condensed tannin (C*alliandra calothyrsus*) or no condensed tannin (lucerne) diets in the presence and absence of either of the worm species *Haemonchus contortus* and *Trichostrongylus colubriformis* to determine any interactions between worms, diet, digesta pH and condensed tannin fractions. The effect of the presence of these nematodes on the concentration of condensed tannin fractions in different segments of the gastrointestinal tract was also investigated. There was a significant ($P \leq 0.005$) diet and worm interaction in the abomasum. Diet had a significant effect on pH values in the abomasum, and the presence of worm infections significantly affected pH values in the ileum, caecum and colon. The presence of worms had no effect on total or fractions of condensed tannin in any of the gastrointestinal segments. The presence of worms had a significant effect on the concentration of all fractions of condensed tannin and total condensed tannin for overall means (pooled for worm species and no worms) in gastrointestinal segments. Protein-bound, fibre-bound, free and total condensed tannin changed significantly along the gastrointestinal tract as influenced

by segments. The relationship between pH and free condensed tannin ($R^2 = 0.196$) and also between pH and total condensed tannin ($R^2 = 0.171$) was correlated.

The protein-condensed tannin complex is possibly the most important condensed tannin complex with respect to nutritional interactions in the gut and anthelmintic effects. The greatest percentage of protein-bound condensed tannin in the gastrointestinal tract was found in the rumen; therefore, condensed tannin could potentially complex with dietary proteins, rumen microbes and nematode third stage larvae. The majority of condensed tannin is in the free form in the abomasum and duodenum, which are the segments inhabited by adult nematodes. A higher portion of condensed tannin was protein-bound in the ileum and possibly the jejunum (extrapolation from pH values), suggesting that there would be significant amounts of condensed tannin bound to dietary protein, digestive enzymes or nematode eggs.

It was also evident that the condensed tannin in the ingested feed could not always be detected in the digesta samples. The total condensed tannin concentration in feed and abomasum were comparable, however, the concentration detected in all other gastrointestinal segments were lower.

In vitro assays were conducted to examine binding and dissociation of condensed tannin extracted from *Calliandra calothyrsus*, with Rubisco and cellulose, at pH 1-9 and from 0.5 to 24 h of complexing without the influence of digesta and gastrointestinal secretions. Complexing time had no significant effect on condensed tannin binding. This study confirmed that *in vitro* binding of condensed tannin with and dissociation from protein is pH dependant; however, this was not the case for the fibre-bound condensed tannin complexing. The pH range at which protein formed the most stable complex with condensed tannin was from 3-9.

Enzyme inhibition studies demonstrated that *Calliandra calothyrsus* condensed tannin does inhibit the activity of the digestive enzymes, trypsin (R2= 0.965) and pancreatic α-amylase (R2= 0.903) with the relationship being well correlated.

The *in vitro* assays demonstrated that *Calliandra calothyrsus* condensed tannin is effective at inhibiting egg hatching, first stage larvae feeding and infective third stage larvae exsheathment and motility in *Haemonchus contortus* susceptible, *Haemonchus contortus* macrocyclic lactone resistant and *Trichostrongylus colubriformis* susceptible strains. The implications are that Callinadra condensed tannin has anthelmintic potential to disrupt the lifecycle of *Haemonchus contortus* and *Trichostrongylus colubriformis*. The eggs and larvae were sensitive to low condensed tannin concentrations with the condensed tannin concentrations demonstrated to have *in vitro* effects well below that found in the GI segments of lambs fed a 100% *Calliandra calothyrsus* diet. There were differences in the effect of *Calliandra calothyrsus* condensed tannin to the different worm species for egg hatch and the larval exsheathment assays only, with the *Haemonchus contortus* susceptible strain being more sensitive than the *Haemonchus contortus* macrocyclic lactone resistant and *Trichostrongylus colubriformis* susceptible strains. *Calliandra calothyrsus* condensed tannin had a greater effect on the *Haemonchus contortus* macrocyclic lactone resistant strain for the larval exsheathment assay. The mimosa extract only had effects on egg hatching and first stage larvae feeding.

The mechanism by which the condensed tannin is having *in vivo* anthelmintic effects by reducing egg production (Cresswell, 2007) may well be due to the effect of condensed tannin on the larvae in the rumen. Alternatively condensed tannin may also complex with the adult nematodes living in the abomasum and duodenum. The *Haemonchus contortus* are abomasal blood suckers and would not be ingesting condensed tannin directly, however, the duodenal grazer, *Trichostrongylus colubriformis* would be ingesting condensed tannin and so may be having an effect via ingestion. Although it was shown that condensed tannin was mostly found as free condensed tannin in the abomasum and duodenum, there were small percentages of protein-bound condensed tannin which, as seen in *the in vitro* complexing, digestive enzyme and egg and larvae assays that condensed tannin is capable of exerting large effects with small concentrations, so this possibility must not be overlooked.

Calliandra calothyrsus condensed tannin is capable of disrupting different stages of the lifecycle *in vitro* of *Haemonchus contortus* susceptible, *Haemonchus contortus*

macrocyclic lactone resistant and *Trichostrongylus colubriformis* susceptible strains. However, not all results from *in vitro* studies can be correlated with what happens *in vivo* due to the physiological conditions present in the gastrointestinal tract and the interactions with constituents of digesta and secretions. Egg hatching, first stage larvae feeding and infective third stage larvae exsheathment and motility were all inhibited due to the effects of *Calliandra calothyrsus* condensed tannin.

Calliandra calothyrsus condensed tannin complexing dynamics does have nutritional and anthelmintic effects. These studies have shown conditions in the rumen and post duodenum to be conducive to condensed tannin complexing with proteins and therefore the potential to complex with dietary proteins, rumen microbes, digestive enzymes and nematode larvae. By-pass proteins formed in the rumen and protein-condensed tannin complexes post duodenum, would be contributing to increased faecal nitrogen output and decreased dry matter digestibility as reported by Cresswell (2007).

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CHAPTER 1 GENERAL INTRODUCTION

The global ruminant livestock industry is very large. It is difficult to put a dollar value on this industry; however, it is easier to look at Australian figures to get some perspective on the size and value of this industry. Australia has a AU$17.4 billion ruminant industry and is the second largest wool producer in the world (approximately 19% of world production) FAOSTAT (2013), however, Australia's sheep, cattle and goat numbers are relatively small at 6.2%, 1.9% and 0.4 % of the total world livestock numbers, respectively.

A significant problem in the ruminant production industry is gastrointestinal (GI) parasitism with infection rates varying from subclinical (often not detectable) to clinical, leading to severe production losses and death. The control of these parasites has many productivity benefits including improvements in weight gain, milk production, feed conversion, reproductive performance and carcass quality as well as possible reductions in morbidity and mortality. Nematode parasites are generally perceived as a greater problem in the sheep/goat industry than the cattle industry due to *Haemonchus contortus* (*H. contortus*), a pathogenic abomasal blood-feeder and to a lesser extent *Trichostrongylus colubriformis* (*T. colubriformis*), a small intestinal grazer that can cause black scours, both having the ability to cause large numbers of deaths compared to very few deaths in cattle. Nematodes cause similar reductions in weight gains in sheep and cattle, with sheep also having decreased wool production (Barger 1982).

The economic losses to the Australian sheep industry due to internal parasites was AU$369 million in 2006 (Sackett et al. 2006), with these figures expected to be higher today. This loss includes both the cost of control (13%) and cost due to losses of meat and wool production as well as mortalities (87%).

There are many non-chemical and chemical management strategies to overcome internal parasitism including rotating cattle and sheep on pasture, breeding programs to incorporate indigenous "resistant" breeds into the gene pool, monitoring faecal

egg counts (FEC) and the use of commercial anthelmintics. A promising practical farm strategy is also to use the clinical evaluation of anaemia (FAMACHA© system), and body condition scoring, resulting in drenching of only those animals unable to cope with the worm burden (Van Wyk and Bath, 2002).

However, of major concern to the ruminant industry is the increasing reported incidences of resistance by GI worm parasites to the range of commercially available anthelmintics. The first reports of anthelmintic drug resistance occurred nearly sixty years ago (Drudge et al. 1957). Resistance is now widespread (Table 2.13) and of alarming concern, particularly to the sheep and goat industries. We now have the extreme situation with resistance to all broad-spectrum anthelmintics in the tropical/sub-tropical small ruminant industry in southern Latin America (Waller et al. 1996), South Africa (Van Wyk et al. 1999), and the southern United States (Howell et al. 2008). Anthelmintic resistance has been most commonly detected in GI nematodes of ruminants, particularly sheep and goats.

Natural anthelmintics found in plants is an area receiving much attention. This has the potential to offer a cost effective and an environmentally friendly alternative, which is especially of interest in developing countries and to organic farmers. Initial indications from work with temperate species *Lotus corniculatus*, *Lotus pedunculatus* (Robertson et al. 1995; Neizen et al. 1998a, Molan et al. 2002; Molan and Faraj, 2010), *Onobrychis viciifolia* (Paolini et al. 2004; Novobilsky et al. 2011) and *Hedysarum coronarium* (Neizen et al. 2002; Aissa et al. 2015) and tropical species *Lespedeza cuneata* (Burke et al. 2012), *Desmodium intortum* (Debela et al. 2012) and the shrub legume *Calliandra calothyrsus* (calliandra) has shown these plants to have an anthelmintic effect (Cresswell 2007). These plants have a high concentration of condensed tannin (CT), which is the suspected agent responsible for the anthelmintic effects.

Past and present studies at James Cook University and internationally, with respect to ruminant animal nutrition in the tropics, has identified two promising shrub legumes, calliandra and *Gliricidia sepium* as high protein sources, with the potential as substitutes for the high quality *Leucaena leucocephala.* Studies here at James Cook

University have largely defined the nutritional values of calliandra and *Gliricidia sepium*; including among others, rates of degradation of dry matter (DM) and protein within the rumen; the resultant short chain fatty acid profiles from these, their gluconeogenic potential and the value of their amino acids for protein synthesis. Calliandra was observed to have a very high quality amino acid profile (Widiawati and Teleni 2004) although a significant proportion of the protein from the legume is excreted as faeces. It is suspected that the cause of such a loss is the high tannin content of calliandra. The potential for further improving the nutritional value of this legume together with its possible anthelmintic properties (high tannin) give calliandra added dimension as a feed. These plants are well suited to the long dry winters and wet summers in the tropics. Coincidently, these tropical species, especially calliandra have also been shown to contain high levels of the CT. Because of this, work at James Cook University started in the College of Public Health, Medical and Veterinary Sciences investigating the anthelmintic properties of some of these shrub legumes. Promising work so far, has shown that it appears the legume has a negative effect on parasites by decreasing egg production (Cresswell 2007). The mechanisms by which the tannin are having an effect are unknown. Therefore, by investigating some of the dynamics both *in vitro* and *in vivo* some of these actions may be elucidated.

Condensed tannin complexing also occurs with proteins and is of interest in the feeding of animals on the CT diets. The protein-CT complex does not allow the plant protein, Rubisco, to be released in the gut for utilization by the animal. Protein is excreted in the faeces bound to the CT (Cresswell 2007). Concomitant with this complexing of Rubisco is the potential for CT to form complexes with and inhibit key digestive enzymes, adding to the inability of the animal to fully utilize proteins. There is the potential to increase the protein availability to the animal from the high tannin plants by elucidating the complexing mechanism.

This study was conducted in conjunction with the PhD of Cresswell (2007), in which the lambs were shared but different parameters were measured in each of the theses. Any results from the findings of the effects on nematodes that Cresswell (2007) found, as well as any nutritional findings between the lambs fed a diet containing

high CT, can be cross-referenced to this current work on CT binding dynamics. The study by Cresswell (2007) did show decreased egg production in lambs fed the tropical shrub legume calliandra, by 64-84% and 24-68% in *H. contortus* and *T. colubriformis*, respectively. A reduction of 15 % dry matter digestibility (DMD) and an increase of 52% faecal nitrogen (N) output was also reported (Cresswell 2007) in lambs fed the high CT diet.

Objectives of the study

The objectives of this research were to:

- conduct large scale extraction and purification of CT from calliandra and to report the percentage of HT and protein present in the resulting purified CT.
- compare the CT assays of Terrill et al. (1992b) and Perez-Maldonado (1994) to determine which of the two methods is best suited for routine analysis of both plant material and digesta.
- examine the dynamics of binding and dissociation of CT in the GI tract of lambs to determine if pH of digesta and infection with worms has an effect on the complexing.
- determine pH of the different segments of the digestive tract and the concentration of the CT fractions (protein-bound, fibre-bound and free-CT) in digesta of lambs fed calliandra or lucerne pellets (pH only) and infected with or without nematodes.
- extract and purify Rubisco from spinach leaves.
- determine pH range and complexing times at which protein and fibre complexes with and dissociates from calliandra CT *in vitro.*
- determine maximum activity and any inhibition of the trypsin and pancreatic α-amylase in the presence and absence of purified calliandra CT.
- determine the *in vitro* anthelmintic effects of purified calliandra CT and mimosa extracts from black wattle.

It should be noted that Keryn Cresswell, a fellow PhD student, who investigated the anthelmintic effects of tropical shrub legumes in sheep, conducted the Experiment referred to in chapter 5. The results of her studies are contained within her PhD thesis.

CHAPTER 2 REVIEW OF LITERATURE

2.1 Tannin

Tannins are naturally occurring water-soluble plant polyphenols (secondary plant metabolites), which have the ability to bind and precipitate proteins. They are widely distributed in plants and are located in the vacuoles or surface wax where they do not interfere with plant metabolism. The term tannin is derived from the Celtic word for oak, which was the traditional source of tannin used to tan hides. These water-soluble polymers are rich in phenolic groups and are capable of binding and/or precipitating water-soluble proteins (Bate-Smith 1973). Many water soluble phenolics, however, do not precipitate proteins and some phenolics are not water soluble (Bate-Smith 1973). The word tannin specifically means that they can precipitate protein, which sets them apart from the phenolics. Apart from protein, tannin can also form complexes with minerals and carbohydrates, including cellulose, hemicellulose and pectin (Price and Butler 1977).

The more widely accepted definition of tannin appears to be:

> any phenolic compound that of sufficiently high molecular weight containing sufficient phenolic hydroxyls and other suitable groups (i.e. carboxyls) to form effectively strong complexes with protein and other macromolecules under the particular environmental conditions being studied (Horvath 1981).

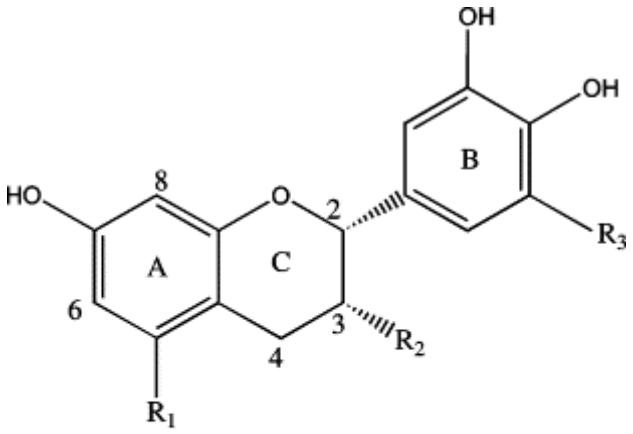
Tannins can be classified into the following types:

- Proanthocyanidins, commonly called CT
- Hydrolysable tannin (HT)
- Oxytannins – readily formed on injury of the plant
- β-tannins – miscellaneous low molecular weight compounds
- Prototannins – tannin precursors eg Flavan-3-ols

Condensed tannin and HT are the two major types of tannins according to their polymer subunit structure and chemical reactivity. The CT are so named due to their condensed chemical structure, however, HT can also be condensed.

2.1.1 Proanthocyanidin or condensed tannin

Proanthocyanidins or CT are polymeric flavanoid-based compounds linked through interflavan carbon-carbon bonds (usually C4-C8 or C4-C6). The generalized structures for Flavan-3-ols and example of proanthocyanidins are shown in Figures 2.1 and 2.2.



R_1	R_3	Class
OH	H	Proanthocyanidin
OH	OH	Prodelphinidin
H	H	Profisetinidin
H	OH	Prorobinetinidin

Figure 2.1 The basic repeating unit in condensed tannins. The structure is that for (-)-epicatechin. The groups at R1 and R3 for other compounds are indicated below the structure. R2 = O-galloyl in the catechin gallates. From Schofield et al. (2001).

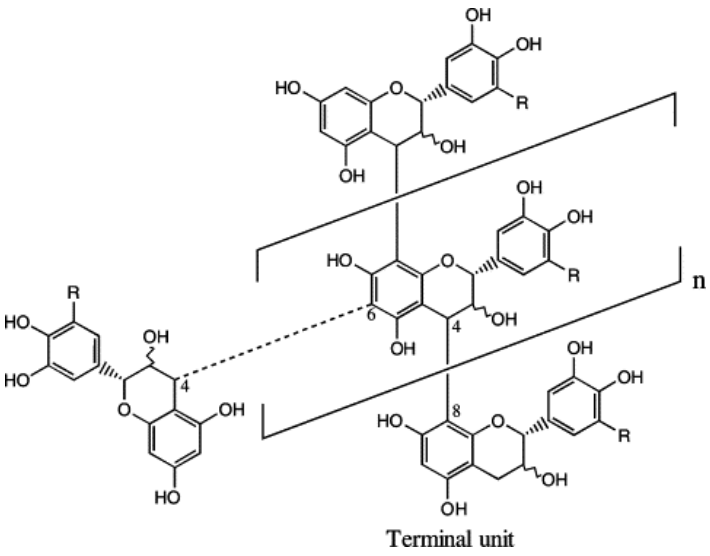


Figure 2.2 Model structure for condensed tannin. If R = H or OH then the structure represents a procyanidin or prodelphinidin. The 4→6 linkage (dotted line) is an alternative interflavan bond. The terminal unit is at the bottom of such a multi-unit structure. From Schofield et al. (2001).

They are so called proanthocyanidins because they yield anthocyanidin pigments upon oxidative cleavage (NOT hydrolysis) after heating in hot alcohol solutions, e.g., acid butanol chemistry (Porter et al. 1986), (Figure 2.3).

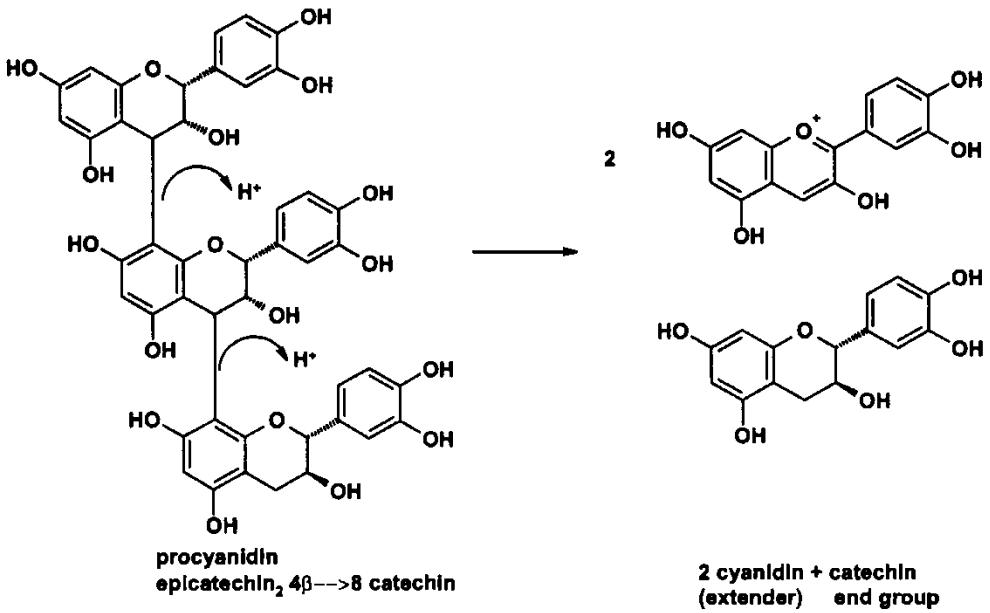


Figure 2.3 Oxidative cleavage of procyanidin. From Hagerman (2002).

The acid butanol reaction results in an unchanged terminal unit and anthocyanidins from the extender units. They are of varying composition and molecular weights ranging from 1900 to 28 000. Not all CT are soluble in aqueous organic solvents,

depending on their structure and degree of polymerisation. Condensed tannins are the most widely distributed in vascular plants and can be found in wood, leaves, seeds and flowers. The anthocyanidin pigments can range from pink, scarlet, red, mauve, violet and blue in flowers, leaves, fruit juices and wines and are responsible for the astringent taste in wines and unripe fruit. The biosynthetic pathway of proanthocyanidin is shown in Figure 2.4.

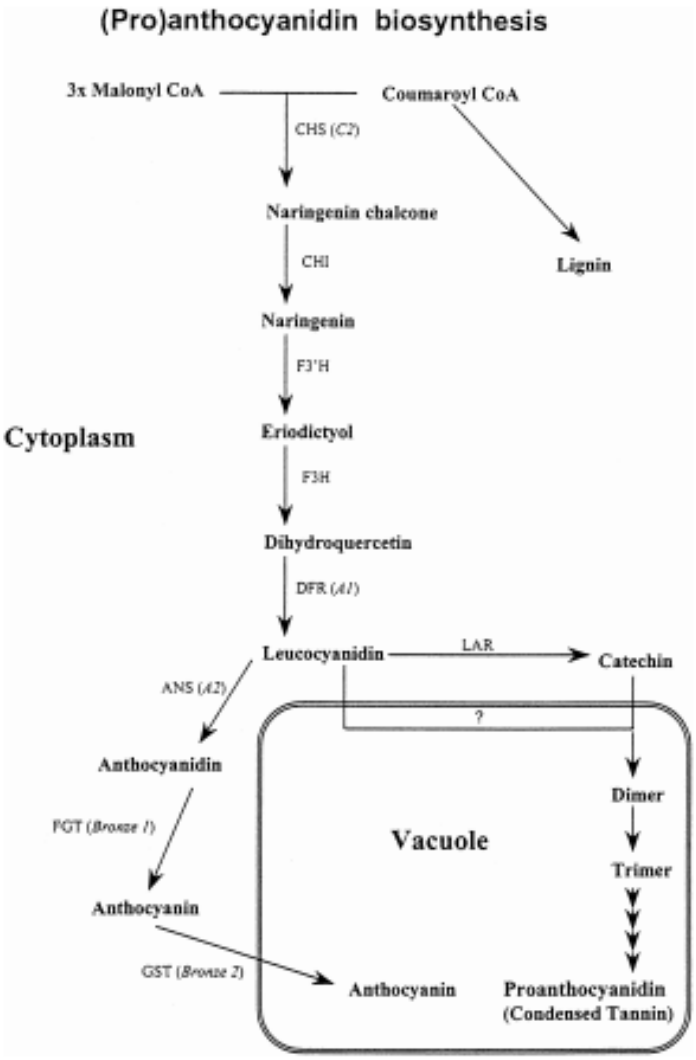


Figure 2.4. Pathways of procyanidin and anthocyanidin biosynthesis. CHS: chalone synthase; CHI: chalone isomerase; F3'H: flavanone-3'-hydroxylase; F3H: flavanone-3'-hydroxylase; DFR: dihydroflavanol reductase; FGT: flavanol-UDP-glucosyl transferase; LAR: Leucocyanidin reductase; ANS: anthocyanidin synthase;?: the proposed CT condensing enzyme [from (Aerts et al. 1999)].

The structure of proanthocyanidins, as shown in Figure 2.5, is based on a heterocylic ring system derived from phenylalanine (B) and polyketide biosynthesis (A). The proanthocyanidin may contain from two to fifty or more flavanoid units.

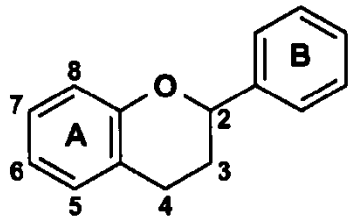


Figure 2.5 Proanthocyanidin flavanoid skeleton and numbering system. From Hagerman (2002).

The proanthocyanidin can be divided into four groups according to structural and chemical reactivity differences.

2.1.1 Flavan-3-ols (epicatechin and catechin)

The most common proanthocyanidins are procyanidin and prodelphinidin and these are also the most widely studied. Derivatives of flavan-3-ols are found in 80% of pigmented leaves, 69% of fruits and 50% of flowers (Swain 1976). The flavan-3-ols are based on (-)-epicatechin and (+)-catechin (Figures 2.6 and 2.7) polymers, which yield cyanidin and are therefore called procyanidins. The addition of a third phenolic group on the B ring produces gallocatechin and epigallocatechin based polymers, which yield delphinidin. There is also a rare mono-substituted flavan-3-ol based polymer, which yields pelargonidin.

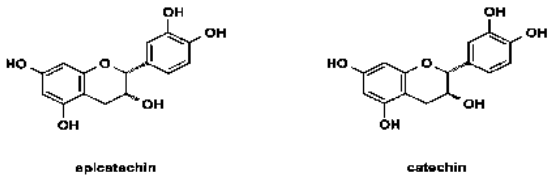


Figure 2.6 The basic flavanoid units of the condensed tannin subunits, epicatechin and catechin. From Hagerman (2002).

Dimers and polymers of catechin and epicatechin are most commonly linked via a carbon-carbon bond between the C8 of the terminal unit and C4 of the extender. The

four most common dimers and couplings are shown in Figure 2.7. If polymerisation continues a linear 4, 8 polymer such as sorghum procyanidin occurs, as seen in Figure 2.8. Other linear polymers based on 4, 6 dimers are less common as with the branching polymers containing 4, 6 and 4, 8 linkages (Figure 2.9).

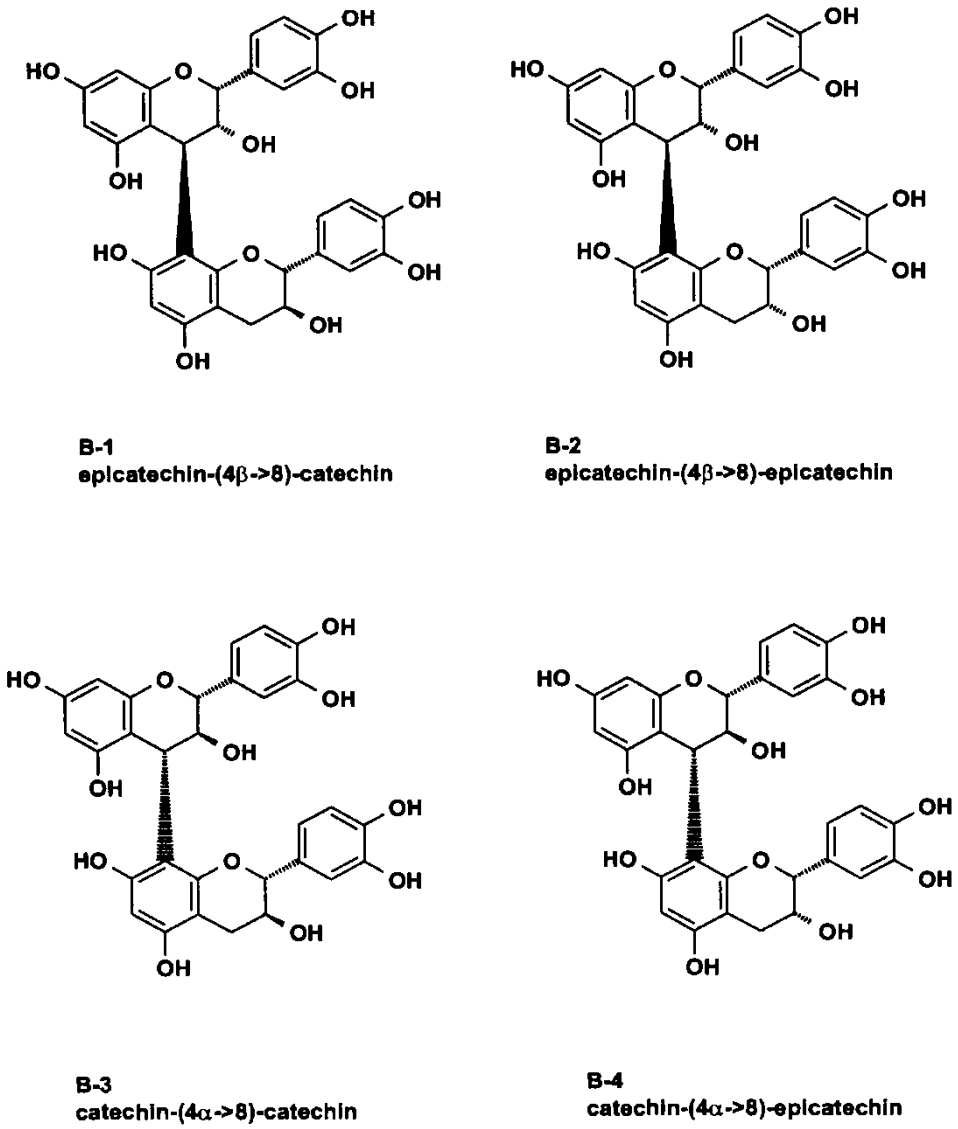


Figure 2.7 The four common C8-C4 linkages of the dimers of the condensed tannin subunits epicatechin and catechin. From Hagerman (2002).

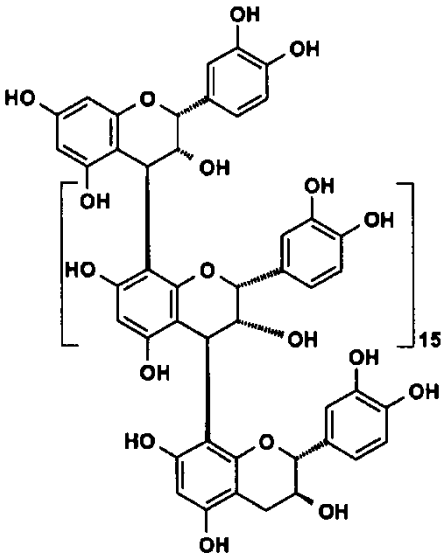


Figure 2.8 The linear 4,8 polymer sorghum procyanidin. From Hagerman (2002).

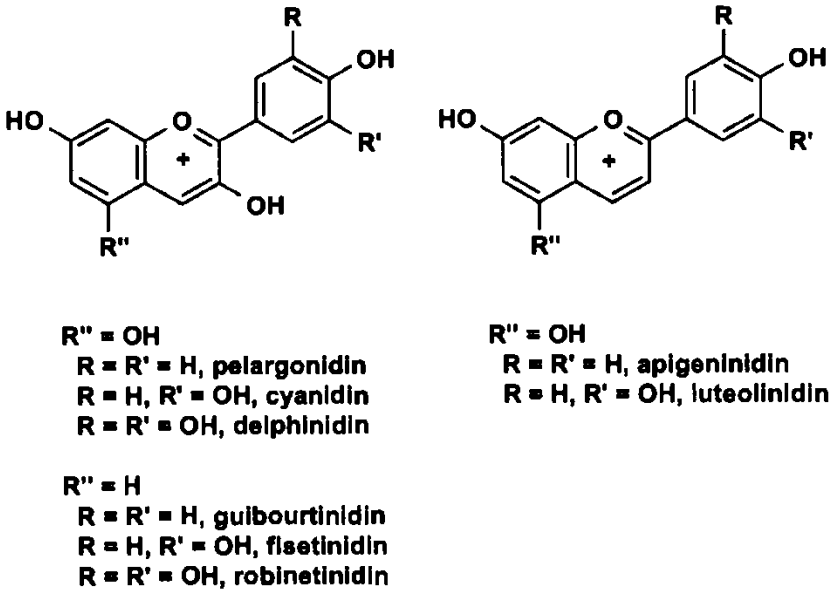


Figure 2.9 Structure of other Flavan-3-ol flavanoid units. From Hagerman (2002).

2.1.2 5-deoxy-flavan-3-ols

The 5-deoxy-flavan-3-ols are commonly branched due to the reactivity of the 5-deoxy A ring and include profisetinidin (Figure 2.10) and prorobinetinidin (the major

tannin in Quebracho and Acacia tannin preparations) which yields the 5- deoxy anthocyanidins fisetinidin and robinetinidin.

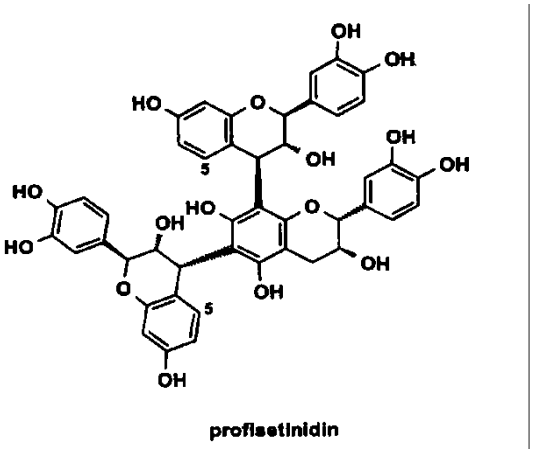
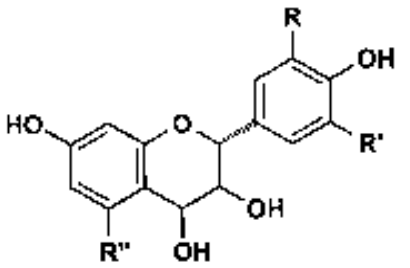


Figure 2.10 The structure of the branched proanthocyanidin profistinidin. From Hagerman (2002).

2.1.3 Flavan-3, 4-diols (leucoanthocyanidins)

The flavan-3, 4-diols are monomeric flavanoids (Figure 2.11) that react with acid and heat as do proanthocyanidin but do not interact with and precipitate proteins.



Flavan-3,4-diols

R" = H (stable)
R = H, R' = OH, leucofisetinidin
R" = OH (unstable)
R = R' = H, leucopelargonidin
R = H, R' = OH, leucocyanidin
R = R' = OH, leucodelphinidin

Figure 2.11 The structure the Flavan-3,4-diol subunits. From Hagerman (2002).

2.1.4 Flavan-4-ols (also leucoanthocyanidins)

These proanthocyanidins yield anthocyanidin upon treatment with alcoholic acid at room temperature**,** (Figure 2.12).

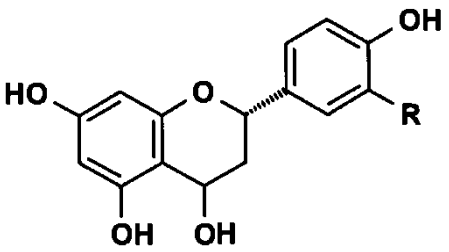


Figure 2.12 The structure of the Flavan-4-ol subunit. From Hagerman (2002).

2.1.2 Hydrolysable tannins

Hydrolysable tannins are derivatives of gallic acid (3, 4, 5-trihydroxyl benzoic acid) with a core polyol, usually glucose, and are found in low concentrations in plants (Haslam, 1989). They are of low molecular weight (500-3000) and are hydrolysed by mild acid or bases and by hot water or enzymes to yield carbohydrate and phenolic acids. Hydrolysable tannin is usually highly toxic to non-ruminants but less toxic to ruminants because they are able to be degraded by acid or enzymatic hydrolysis in the rumen. There are 2 major types of HT:

2.1.2.1 Gallotannins

The gallotannins, for example, tannic acid, are the simplest HT and made up of polygalloyl esters of glucose (Figures 2.13 and 2.14). The molecule is flat and disclike and is able to be hydrolysed with a strong acid producing gallic acid and the core polyol (Haslam, 1989).

There must be at least three hydroxyl groups of the glucose esterified to enable sufficient protein binding to be classed as tannin.

2.1.2.2 Ellagitannins

Simple ellagitannins are esters of hexahydroxydiphenic acid which spontaneously lactonises to ellagic acid in aqueous solution, as seen in Figure 2.14.

Ellagitannin molecules may be much more spherical in shape than the gallotannins.

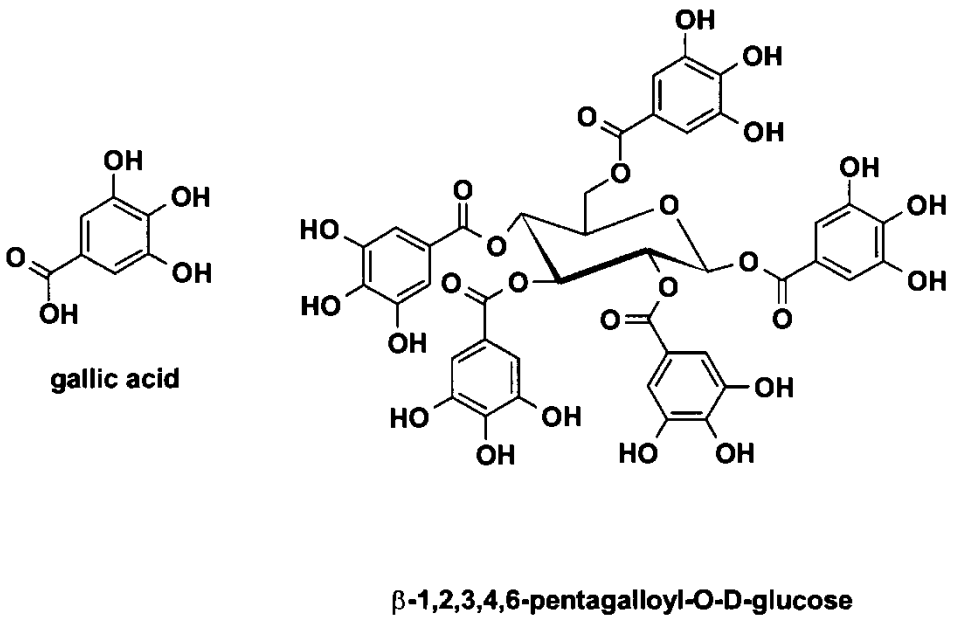


Figure 2.13 Structure of a gallic acid unit and a gallotannin (β-1,2,3,4,6-pentagalloyl O-D-glucose). From Haslam (1989).

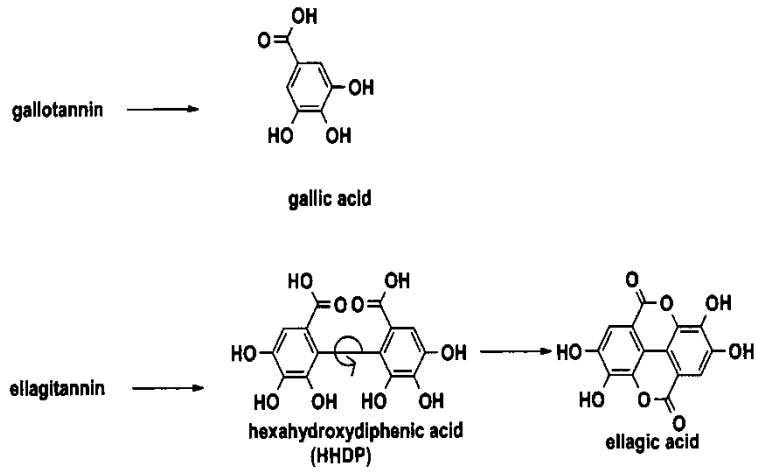


Figure 2.14 Structure of the gallotannin and ellagitannin subunits. From Haslam (1989)

2.1.3 Tannins in plants

Tannins are found in fruits, tea, chocolate, legume forages, legume trees and grasses. In general, tannins are considered to be the basis of plant chemical defense mechanism against pathogens and herbivores. Tannins found in the buds appear to

aid resistance to freezing, in leaves to reduce palatability and in the stem, roots and seeds as an antimicrobial agent. Tannins are more commonly found in Dicotyledons than Monocotyledons and are responsible for the wide range of colours found in flowers and leaves. The Monocotyledons contain only CT, while the Dicotyledons may contain either CT or HT or both (Bate-Smith 1977), (Table 2.1). Table 2.2 shows examples of the type of tannin in some plants. Hydrolysable tannins have since been reported to be present in some algae (Nishizawa et al. 1985).

Table 2.1 The distribution of condensed tannin (CT) and hydrolysable tannin (HT) in plants.

	Genera examined containing tannins	
Taxon	CT (%)	HT (%)
Psilopsida	0	0
Lycopsida	0	0
Sphenopsida	28	0
Ferns	92	0
Gymnosperms	74	0
Angiosperms	54	13
Dicotyledons	62	18
Monocotyledons	29	0

(From Swain 1979).

Table 2.2 Tannin types identified in different plants.

Plant source	**Tannin identified**	**Tannin Type**	**References**
Sorgum bicolour (grain)	CT and catechin	CT	(Asquith and Butler 1986)
L. Pedunculatus (leaves)	CT	CT	(Barry and Manley 1986)
L. corniculatus (leaves)	Epicatechin epigallocatechin	CT HT	(Foo et al. 1996)
Acacia nilotica (leaves)	catechin monodigallates	HT	(Self et al. 1986)
Camellia sinesis (green tea)	epicatechin, epigallocatechin gallates	CT HT	(Bradfield and Bate-Smith 1950)
L.leucocephala (leaves)	gallotannins catechins	HT, CT	(D'Mello and Fraser 1981)
Ceratonia siliqua (carob pods)	galloyl-D-glucose, flavan-3-ol-gallates	HT	(Haddock et al. 1982)

Adapted from (Mangan 1988)

Plants high in tannins include the families of *Leguminosae* (*Acacia sp*., *Sesbania sp., lotus sp.,)*, *Anacardiaceae* [*Scinopsis balansae* (Quebracho)], *Combretaceae* (myrobalan), *Rhizophoraceae* (mangrove), *Myrtaceae*, *Eucalyptus sp., Mirtus sp.* (Myrtle), *Polinaceae* (*canaigre*). Other species worth mentioning for their tannin content are oak, maple, willow, pine and *Sorghum sp.*

2.1.4 Tannin-protein complexes

The details of the tannin-protein complexes are only partly understood. The free phenolic hydroxyl groups form strong hydrogen bonds with proteins and carbohydrates (Haslam 1989). Hydrogen bonds are one of three non-covalent, secondary or weak bonds that can occur in biochemical systems that are strong enough to form structures or complexes and weak enough to be readily dissociated. Binding with small numbers of hydrogen bonds are easily reversed but larger numbers having the ability to produce intra and intermolecular interactions that are stable. These bonds are due to the electrostatic attraction between the two electronegative molecules oxygen and nitrogen and the electropositive molecule of hydrogen as shown below:

−O−H---O−

−O−H---N−

−N−H---O−

−N−H---N−

The hydrogen bond is of maximum strength when all atoms are in a straight line. The CT-protein complex occurs due to the interaction of hydrogen bonding between the phenolic hydroxyl and the peptide carbonyl (Hagerman 1980, Hagerman and Butler 1980b). This bonding is strengthened when the amide nitrogen adjacent to the carbonyl is alkyl substituted (Hagerman 1980), as with the imino nitrogen in the peptide linkage with proline. Tannins may also complex with proteins through hydrophobic bonding (Oh et al. 1980) and can form covalent bonds with proteins through oxidative polymerisation reactions due to heating, exposure to ultra violet radiation and the action of polyphenol oxidase. The strength of the tannin-protein complex depends on characteristics and chemistry of both the tannin and the protein (molecular weight, tertiary structure, isoelectric point, and compatibility of binding sites). These interactions are also influenced by the reaction conditions (temperature, pH, and tannin: protein ratios). These tannin complexes are reversible and can be

disrupted by detergents or pH (Jones and Mangan 1977). The interactions between CT and proteins are selective and specific, influenced by the size of the proteins and polypeptides. Results from a study by Hagerman and Butler (1981) testing a variety of proteins and synthetic polymers using a competitive binding assay showed that proteins with a molecular weight of less than 20 000 had low affinities for sorghum tannin. Tightly coiled globular proteins have much lower affinities for tannins than conformationally loose proteins, and proline-rich proteins and polymers have a very high tannin affinity. Small polypeptides with residues less than seven have been found to interact very weakly with tannins (Hagerman and Butler 1981), suggesting that this is due to the CT-protein interactions involving multiple binding sites. Increases in the ratio of prodelphinidin:procyanydin have also been shown to influence protein complexing (Aerts et al. 1999).

Proteins are precipitated by CT most efficiently at pH values near their isoelectric point where the protein-protein electrostatic repulsion is minimal (Hagerman and Butler 1981). Osborne and McNeill (2001) tested CT-protein (using CT extracted from different *Leucaena sp* and bovine serum albumin (BSA), with an isoelectric point of pH 4.9) binding capacities at more acidic (pH 2.5) and more alkaline (pH 7.5) than the isoelectric point of the protein. These results showed that increasing or decreasing the reaction pH from pH 5 decreased the binding capacity of the CT from the *Leucaena sp*, with the decrease being highest at the lower pH. Complexes between the plant Rubisco protein (ribulose-1, 5-bisphosphate carboxylase) and CT from sainfoin have been shown to be stable between pH 4.0-7.0, with 30% dissociated from the protein complex at pH 8.0-8.5, and 95% dissociated from the protein complex between pH 1.0-3.0 (Jones and Mangan 1977). The decrease in pH of the abomasum may therefore induce the CT previously bound to protein in the rumen to be released. Jones and Mangan (1977) also found that in rumen samples (pH 6.5) from sheep the CT was tightly bound to the protein, but in the duodenal samples (pH 2.5) the CT was very loosely bound to the protein or some other digesta component. In the same *in vitro* experiments Jones and Mangan (1977), showed that the CT-protein complex dissociated at 39 ºC and not at 0 °C, and when the complex was allowed to age for 24 h the protein release was decreased compared to the complex tested after 2 h.

2.1.5 Current analytical methods to identify and quantitate tannins

The analysis of tannins is difficult owing to the complex chemical structure and diversity of tannins. There is a vast array of analytical techniques to quantify tannins of plant origin. General phenolic assays and specific functional group assays have been developed for the analysis of tannins. Tannins must be extracted from the plant of interest and in most cases purified before analysis. The widely accepted extraction procedure was adapted by Terrill et al. (1990), from that of Broadhurst and Jones (1978). The standard purification method uses sephadex LH -20 (Terrill et al. 1990). This standard extraction and purification method does not separate the CT from the HT, and also contains low molecular weight phenolics (Kraus et al. 2003). Methods commonly used include the acid-butanol assay, vanillin assay, precipitation reactions, enzyme and microbe inhibition assays, gravimetric techniques and HPLC.

2.1.5.1 Colorimetric analysis for total phenols

Various techniques for the analysis of total phenolics have been published. These assays are not specific for particular phenolic compounds but quantify total concentrations of phenolic hydroxyl groups in the plant extract of interest. The Folin-Ciocalteau (Folin and Ciocalteu 1927) and Prussian blue method of Price and Butler (1977) as modified by Graham (1992) are the most popular methods. The Folin-Ciocalteau (Folin and Ciocalteu 1927) method is an improved version of the Folin-Dennis method and measures tyrosine in proteins and will react with all phenolics. The Prussian blue method (Price and Butler 1977) has been since modified by Graham (1992) and also measures total phenolics. This is the most popular method as it is a simple and rapid technique.

Functional group assays

Functional group assays measure specific molecular structures and can therefore distinguish between HT and CT structures.

2.1.5.2 Assays for CT

The structural complexity of CT makes their study difficult. Condensed tannin can exist in plants as protein-bound, fibre-bound and unbound (free) fractions. There are two common colourimetric methods used to assay for the extractable or unbound fraction of the CT.

2.1.5.3 Vanillin assay

The vanillin-HCl method of Burns (1963) is based on the reaction of vanillin with CT and is specific for flavanols, however, has unreliable reproducibility, is difficult to standardize and only assays for a narrow range of flavanols and dihydrochalcones. This assay reacts with the A ring of the CT to produce a coloured product, with absorbance intensifing with a decreasing molecular size. The chemistry of the assay can be seen in Figure 2.15.

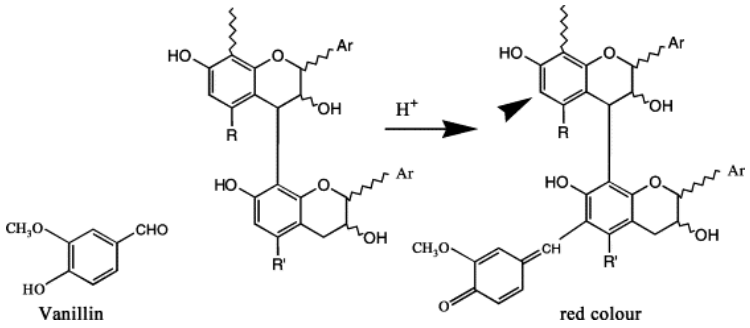


Figure 2.15 Chemistry of the vanillin assay for condensed tannins. The arrowhead points to a second potentially reactive site.

2.1.5.3 Acid-Butanol assay

The acid-butanol assay oxidatively cleaves the polymer subunits, producing a red coloured anthocyanidin. The *n*-butanol-HCl method described by (Porter et al. 1986) is specific for CT, is simpler and gives a good indication of CT concentration compared to the vanillin-HCl assay. The chemistry of the assay can be seen in Figure 2.16.

There have been many different modifications on this method to try to minimize some of the limitations to this assay, including water and transition metal interference, ease of cleavage by acid, number of phenolic groups affecting wavelength maximums and non-linearity at higher CT concentrations.

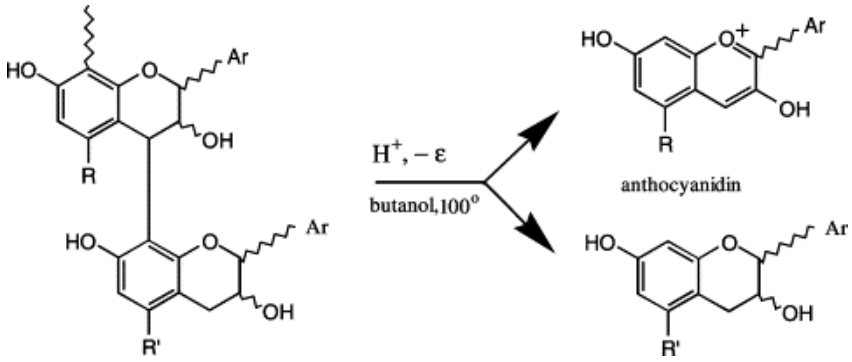


Figure 2.16 Chemistry of the acid–butanol reaction. Note that the reaction involves oxidation and that the terminal unit does not give a colored anthocyanidin product structure.

It has been reported that the amount of water in the assay may change the colour development when different plant species were used (Dalzell and Kerven 1998). However, Hagerman and Butler (1994) reported that as long as the standards contained the same amount of water the assay would produce satisfactory results. Procyanidin content of the CT seems to affect the assay, with a higher procyanidin content yielding a higher absorbance in the assay for the same tannin concentration (Kraus et al. 2003). This is in contrast to reports that there are no differences in the yield due to procyanidin and prodelphinidin composition of the extract (Porter et al. 1986). Colour yield was also reported by Mole and Waterman (1987) to be non-linear at higher CT concentrations and due to interaction of transition metals, they have now eliminated transition metals from this method Terrill et al. (1992b).

2.1.5.4 Quantitation of condensed tannin fractions

The protein-bound, fibre-bound and unbound portions of the CT are able to be assayed using the modified method of Terrill et al. (1992b), based on the original *n*-butanol-HCl method of Porter et al. (1986), which incorporates an acetone-water-diethyl ether extraction and sodium dodecyl sulphate (SDS) extraction. Terrill et al. (1992b) reports that this method appears to be the most accurate at CT concentrations of 1% or greater. This method has since been modified by Perez-Maldonado (1994), for analysis of digesta and faecal samples, as the author claims the method of Terrill et al. (1992b) was not sufficient in dissociating the CT from the protein, however, there still appears to be limitations to this method. For the protein-bound fraction, the pH has been changed from 8.0 to 10.0 by replacing the tris-HCl buffer (HCl was found to interfere with the assay) with tri-ethanolamine, as well as using a much

lower concentration of 2-mercaptoethanol to make the procedure safer in the laboratory. The fibre-bound tannin assay was determined in methanol instead of water and a final extractant of ethyl acetate was added to the free-tannin estimation to eliminate small phenolics. Comparative results from these two methods have not been published.

2.1.5.5 Assays for hydrolysable tannin

Several assays have been developed for the determination of HT. The rhodanine assay reported by Inoue and Hagerman (1988) can detect the gallotannins. There are also assays for detecting gallotannins plus ellagitaninns (Hartzfeld et al. 2002). There is an assay to detect ellagitaninns only that has more recently developed (Wilson and Hagerman 1990). Hydrolysable tannin can be measured using high pressure liquid chromatography (HPLC), however, it is difficult to analyse the large CT polyphenolics by HPLC (Hagerman 2002).

2.1.5.6 Gravimetric methods

Gravimetric methods have been described by Makkar et al. (1993) and Reed et al. (1985) for quantification of phenolics in plant material. Standards are not required for these methods. The method described by Reed et al. (1985) utilizes trivalent Ytterbium to precipitate phenolics while the method described by Makkar et al. (1993) measures tannins bound to insoluble polyvinylpyrrolidone.

2.1.5.7 Precipitation assays

Protein-binding assays can be used to determine both biological activity and amount of total tannins in a sample using the crude plant extract. Precipitation assays are useful in predicting the negative effects that tannins may have in plants with respect to ruminal degradation (Silanikove et al. 1996). Protein precipitation capacity can be measured by a simple Radial Diffusion Assay (Hagerman 1987). The amount of protein precipitated being directly proportional to the amount of total tannin in the sample. An additional step can be added to destroy the HT in the sample so the proportional activity of CT can be calculated. The proteins commonly used in this assay are Bovine Serum Albumin (BSA) and gelatin, which may not behave in the same manner as the plant protein.

Polyethylene glycol (PEG) is a non-ionic detergent with a molecular weight of 4000 which forms complexes with both HT and CT from a pH range of 2.0-8.5 (Jones 1965). Silanikove et al. (1996) reported using a PEG binding assay in situ without the need for extraction. An advantage of the PEG binding assay over the protein precipitation assay as it is able to be conducted on plant samples that have been dried at 90 ºC.

2.1.5.8 Other assays

Nuclear magnetic resonance spectroscopy, mass spectrometry, Gel Permeation Chromatography and both normal and reverse phase HPLC techniques are some alternative methods used in detection and characterization of tannins. Assays have been developed to measure polymer length, molecular weight and ratios of procyanidin to prodelphinidin and other anthocyanidins (Williams et al. 1983, Hammerstone et al. 1999, Lazarus et al. 1999). Nuclear magnetic resonance spectroscopy can be used to identify the proportion of HT and CT in a plant extract. The spectra can be used to determine the ratio of procyanidin to prodelphinidin only if there is no HT present in the sample as HT produces an interfering peak at the same chemical shift (Kraus et al. 2003). Structural composition of the plant extract (CT and HT) and the procyanidin to prodelphinidin ratios of the CT appear to have an impact on chemical reactivity (Kraus et al. 2003). Modern techniques using LC-ESI-NMS are revealing a more accurate analysis of tannins. The use of LC-MS together with the development of high resolution TOF-MS equipment is useful in identifying previously unknown tannin structures (Salminen et al. 2011). These techniques are being used to characterise the activity of tannins due to tannin oxidation (Salminen et al. 2011).

Thiolysis and the Butanol-HCl-Iron assay are also proving useful for the determination of CT. Thiolysis is the reaction that occurs when CT is heated in the presence of acid and either benzyl mercaptan (Matthews et al. 1997; Labarbe et al. 1999; Gea et al. 2011) or Phloroglucinol (Matthews et al. 1997). This would essentially allow both chain length and composition of the CT to be determined by HPLC. The Butanol-HCl-Iron assay (Grabber et al. 2013) is a modification of the

Butanol-HCL assay using a Butanol-HCL iron reagent containing acetone as the solvent, which was found to enhance anthocyanidin yields.

2.1.5.9 Enzyme activity and inhibition

Tannins have the capability not only to complex with dietary proteins but also digestive enzymes. Enzyme inhibition assays are useful in the determination of the biological effect of the tannin. Assays have been developed for application in both the animal production and food industries. Some of the assays for determining the inhibitory effects that tannins have on enzymes include assays for the activity of lipase, salivary and pancreatic α- amylase, trypsin, alkaline phosphatase, esterase, cellulose and β-glucosidase. The activity of these enzymes is compared in the presence and absence of the suspected inhibitor. The nature of tannin inhibition, competitive, un-competitive or non-competitive, can be characterized by enzyme kinetic studies (Michaelis and Menten 1913).

Trypsin inhibition is commonly measured using a modified version of the method of Quesada et al. (1995), derived from the original method of Kakade et al. (1969). This colorimetric method measures the production of a yellow dye (p-nitroaniline) at 410 nm due to trypsin hydrolysis of the colourless synthetic dye, benzoyl-DL-arginine-p-nitroanilide (BAPNA). The use of BAPNA eliminates any interference with a protein substrate and was first described by Erlanger et al. (1961). This method has also been adapted to measure large numbers of samples using a micro titer plate technique (Fickel et al. 1999).

Pancreatic α-amylase inhibition can be measured using a modified version of the starch-iodine method of Quesada et al. (1995) derived from the original α-amylase activity method of Briggs (1967). Modification to add HCl to stop the enzymatic reaction has since been reported by Xiao et al. (2006).

There are a large number of different *in vitro* assays to measure esterase and lipase activity and hence inhibition of activity by tannins. Lipase assays measure the production of either fatty acids or glycerol or triacylglycerol or fatty acid esters. Some of these methods used to determine lipase and esterase activity include

colourimetric and turbidity assays, titrimetry and chromatography. Colourimetric assays that are routinely used, utilize the release if p-nitrophenol from p-nitrophenol esters as first described by Huggins and Lapides (1947). For the analysis of esterase the butyrate and acetate can be used as the substrate and for lipase activity laurate and palmitate can be used. von Tigerstrom and Stelmaschuk (1989) more recently developed a turbidimetric assay to measure increases in turbidity upon release of fatty acids from lipase hydrolysis, which is more sensitive and simple but does not seem to have been adopted by other laboratories. Titrimetic assays are simple and very reliable, however, the titrations for the end point are laborious and time-consuming (Dole 1956). Chromatographic techniques such as Thin Layer Chromatography, Gas chromatography and HPLC all give a determination of products released after hydrolysis of a lipid by lipase.

Alkaline phosphatase inhibition by tannin is routinely measured using a micro titer plate binding assay (Ittah 1991). This utilizes the ability of tannins to cross link proteins using BSA as the coating on the plate and is used to quantitatively determine tannin content. The amount of alkaline phosphatase bound to the tannin is assayed by addition of p-nitrophenylphosphate substrate and is proportional to tannin in the test solution.

Modifications to this method have been made to assay for tannin in red wine, with casein or gelatin also used as the plate coating (Adams and Harbertson 1999).

Cellulase activity can be measured by the *in vitro* colourimetric technique. This method estimates the amount of reducing sugars produced after incubation with cellulose and tannin (Petersen and Hill 1991).

2.1.5.10 Microbial inhibition

The effects of tannins on rumen microbes can be determined using a variety of techniques which include gel diffusion assays, fluorescence dye techniques, comparing microbial growth in culture with and without tannins, substrate disappearance assays, *in vitro* fermentation and bacteria binding assays. Bacteria for many of these assays are cultured using the anaerobic culture technique described by

Hungate (1950) and later modified by Bryant and Burkey (1953). Further modifications have been reported (Nelson et al. 1997).

Gel diffusion assays using a variety of indicators on agar plates have been described (Lawrence et al. 1967, Stead 1986), however, there were some early problems with this assay which have been reportedly overcome with the use of fluorescence dye techniques (Kouker and Jaeger 1987). The principles of the tannin–microbe binding assays (Jones et al. 1994, Nelson et al. 1997) are similar to the protein-binding assays. A bacterial culture is exposed to tannins the bacterial pellet is analysed for CT using the *n*-butanol-HCl method of Porter et al. (1986) after incubation, washings and spinning. Bacterial growth inhibition can also be used to study the effects of CT. Tannin is added to a bacterial culture and growth is monitored by change in absorbance at 600 nm compared to a culture containing no tannins (Nelson et al. 1997). *In vitro* fibre fermentation techniques (Giner-chavez 1996) have also been reported to be a relative measure of tannin inhibition of fibre fermenting rumen microbes.

2.2 Sample Preservation and Preparation

Fresh plant material generally has low complex formation and polymerisation and has better solubility in aqueous organic solvents (Reed 1995), compared to dried material, however, it is not always practical to cut the plant and directly extract, especially when larger quantities are to be analysed. Plant samples are therefore generally cut and preserved until analysis. The method of sample preservation has been shown (Mould and Robbins 1981, Terrill et al. 1990) to greatly affect the levels of extractable or free CT in the plant sample. Freeze drying or freezing the sample as long as samples are not thawed before analysis seems to be the preferred preservation method. Freeze drying has been shown to result in the highest levels of free CT in sericea lespedeza (*Lespedeza cuneate*) samples that were analysed after either being freeze-dried, sun cured, oven dried or fresh frozen (Terrill et al. 1990). Oven-drying samples has been shown to have variable effects on the tannin content, but in general gives a lower total CT value (Jackson et al. 1996a). Both drying and temporary freezing of the plant tissue before analysis allows cells to disrupt and the free CT to

complex with the proteins or other cell constituents. Heating has also been shown to increase polymerisation, or induce oxidative changes to the CT.

2.3 Standards

A range of standards are reported in the literature for use in CT assays. This is problematic as the tannins may be a mixture of chemically distinct types of tannins and may include both CT and HT (Haslam 1979). This has implications of underestimation or over-estimation of the CT as the standard chosen may not match the CT in the sample being analysed. Cyanidin, catechin, tannic acid, delphinidin and quebracho have been used as standards, just to name a few (Hagerman and Butler 1989, Scalbert 1992, Giner-Chavez et al. 1997). Figure 2.17 shows relationship between absorbance of tannins from different plants and three compounds used as standards, cyanidin, delphinidin and quebracho, with the large differences between the compounds used as standards (Giner-Chavez et al. 1997). From this figure it can be seen that using quebracho as a standard in a plant predominantly cyanidin would grossly under-estimate CT concentration.

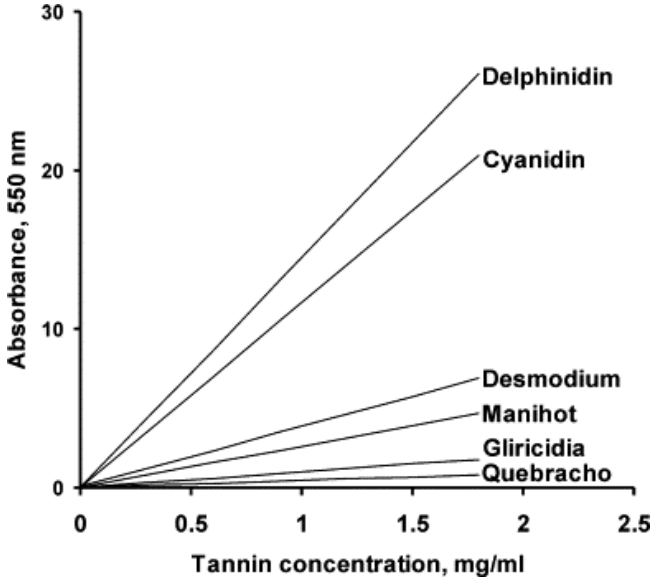


Figure 2.17 Relationship between tannin concentration and absorbance at 550 nm (A_{550}) in the acid–butanol assay. Desmodium: *D. ovalifolium*; Manihot: *M. esculenta*; Gliricidia: *G. sepium*. From Giner-Chavez et al. (1997)

Commercially available standards e.g., tannic acid and catechin, will assay for relative amounts of tannin and must be reported as tannins per weight of the commercial standard (Hagerman and Butler 1989). The Rhodanine and total

phenolics assays can be standardised with gallic acid, the vanillin assay with catechin and the acid-butanol assay with cyanidin. The acid butanol assay if standardised with cyanidin, however, would be only appropriate if the sample contained procyanidin and not for the samples containing prodelphinidin or other CT (Hagerman and Butler 1989). To overcome the problems of an inappropriate standard, using purified extracts from the plant of interest as an internal standard will give an absolute estimate of the tannin being quantified.

2.4 Tropical Shrub Legumes

Past and present studies at James Cook University and internationally, has identified two promising shrub legumes, calliandra and *Gliricidia sepium* (Gliricidia) as high protein sources, with potential as substitutes for the high quality *Leucaena leucocephala*, (Leucaena). These plants are well suited to the long dry winters and wet summers in the tropics. Coincidently, these tropical species, especially calliandra have also been shown to contain high levels of CT (Jackson et al. 1996a). Many studies have been published on the CT content of plants, with values varying due to the assay type and the standards used. Collection and storage conditions can influence the CT values obtained as well as climatic and soil conditions under which the plants were grown. Table 2.3 reports the CT values of three tropical legume species from north and south-east Queensland in Australia and Columbia in South America (Jackson et al. 1996a, Perez-Maldonado and Norton 1996). In this same study CT values from species in Columbia were reported as being much higher in the calliandra, which also varied due to the season harvested.

Table 2.3 Condensed tannin (CT) concentrations of leaf and stem < 2 mm in diameter from *G.sepium, C.calothyrsus* and *L.leucocephala* determined using the Vanillin-HCl (Burns 1963, Broadhurst and Jones 1978) and the *n*-butanol-HCl (Bate-Smith 1973, Porter et al. 1986) methods.

Forage	Condensed Tannin (g/kg DM)					
	Vanillin-HCl		*n*-Butanol-HCl			
	Extractable	Extractable	Protein-bound	Fibre-bound	Total CT	Reference
North Queensland, Australia						
G.sepium	2.6	0.1	37.3	3.3	40.7	1
L.leucocephala	34.6	54.7	4.1	1.5	60.3	1
C.calothyrsus	64.8	50.2	6.2	1.4	57.8	1
South-East Queensland, Australia						
C.calothyrsus	109.3	47.1	12.2	15.7	75.0	2
Columbia, South America						
C.calothyrsus (July)	190.5	158.1	28.4	7.8	194.3	1
C.calothyrsus (Dec.)	214.5	102.9	10.0	3.5	116.4	1

1. Jackson et al. (1996a); 2. Perez-Maldonado and Norton (1996)

2.4.1 *Calliandra calothyrsus*

Calliandra is a small Central American tropical leguminous tree of the subfamily Mimosideae (Wiesum and Rika (1992). Calliandra was originally grown as an ornamental tree and has spreading branches with dark foliage and crimson powder-puff flowers. In 1936 foresters carried seeds from Guatemala to Indonesia where it is now extensively grown for many purposes including firewood, erosion control, shade trees and animal fodder. The plants are easily established from seeds, seedling and cuttings and grow very rapidly to 2.5-3.5 m within 6-9 months. Calliandra can survive in a wide range of soil types and altitudes (150-1500 m) and requires 1000 mm of annual rainfall, but can withstand several months of drought.

2.4.2 *Gliricidia sepium*

Gliricidia is a tropical tree legume native to Pacific coasts and seasonally dry inland valleys of Mexico and Central America (Wiesum and Nita (1992). It is now grown throughout most of the tropics. The leaves being used for animal fodder and green manure, and the wood is used for fuel, charcoal, poles, construction timber and agricultural implements. Gliricidia is important as live fencing, crop shade, soil stabilisation and improvement through nitrogen fixation and has several medicinal uses. Gliricidia can tolerate a wide range of soil types (except acid soils) and climatic

conditions, with rainfalls from 300 mm to 600 mm. The shrub grows very rapidly (up to 6 m in 12 months), resprouts vigorously after lopping and can be easily propagated from cuttings. After flowering, a dry period of 6-8 weeks is required to set seeds. In areas of yearlong rainfall, new plants are propagated from stakes.

2.4.3 Leucaena leucocephala

Leucaena is a branched shrub legume with bipinnate leaves and white flowers and from the subfamily Mimosideae (Jones et al. (1992). It is native to Central America, but can now be found throughout most tropical and subtropical regions. Leucaena will not tolerate a low pH soil or waterlogging and requires an annual rainfall of 750 mm or greater. It was originally used as green manure and shade crop in Indonesia as well as now for animal fodder. Leucaena is difficult to establish, whereafter it has vigorous growth and prolific seed production. The leaflets contain about 5% of the toxic substance mimosine (an uncommon amino acid), which cause cattle to produce lower concentrations of the thyroid hormone, thyroxine.

2.4.4 Nutritive value of shrub legumes

Dry matter digestibility is the proportion of a feed that is able to be digested by the animal or microbial enzymes, and available to be absorbed by the animal (Van Soest 1994). The nutritive value and CT content reported of these tropical species varies as shown in Table 2.4. The crude protein (CP), (CP = nitrogen (N) x 6.25) content of a feed is essential to provide N for the rumen microbial population to synthesise protein, and also additional by-pass protein for direct absorption in the small intestine. For maintenance, ruminants require a minimum dietary CP content of > 8% DM to maintain the rumen ammonia threshold (> 70 mg ammonia N/L) to sustain microbial fermentation and protein synthesis (Satter and Slyter, 1974). Higher dietary CP contents (12-25% DM) are required for animal production (NRC 1995).

Table 2.4 Percentage crude protein (CP), dry matter digestibility (DMD), dry matter (DM) total condensed tannin (CT) and presence of hydrolysable tannin (HT) in the tropical legumes *G.sepium*, *L.leucocephala* and *C.calothyrsus.*

Forage	% CP	% DMD	% DM	% CT
G.sepium	21-24	53-67	22	4
L.leucocephala	14-40	42-70	40	2.5-6
C.calothyrsus	24	22-25	36	6-20

(Felker et al. 1982; Terrill et al. 1992a, Terrill et al. 1992b, Waghorn et al. 1994, Douglas et al. 1995, Jackson et al. 1996a, Perez-Maldonado and Norton 1996, Dalzell et al. 1998, Kang'ara et al. 1998, Merkel et al. 1999)

The CP percent of the three legume species Gliricidia, Leucaena and calliandra, shown in Table 2.4 are high and well within the range for animal production .It can also be seen that as CT concentration increases, with the highest CT concentration found in calliandra, that DMD decreases.

2.5 Tannins in the Gastrointestinal Tract

Binding and dissociation of tannins in the GI tract

Condensed tannin can exist in three different forms in the plant as protein-bound, free and fibre-bound, with these fractions also being interchangeable and reversible along the GI tract. The CT-complex binding and dissociation is influenced by pH, presence of plant and animal proteins, microbes and enzymes, as well as the presence of bile secretions in the duodenum (Jones and Mangan 1977, Hagerman and Butler 1981, Gu et al. 2011, Matsumoto et al. 2011, Niderkorn et al. 2012).

Plant material is masticated in the mouth (and re-masticated during rumination) by the animals releasing some of the tannin from the plant vacuoles. The pH of the rumen (~5.5-6.5) would allow any free tannin to complex with proteins and fibre. Here the CT may inhibit protein degradation by protecting the plant from proteases or by binding to the enzymes to inhibit protease activity. The long period that the digesta spends in the rumen would assist in the complex formation as it appears to be time-dependent (Jones and Mangan 1977). Once the digesta flows to the abomasum the CT complexes formed in the rumen would be able to dissociate producing free CT if the pH was below three (Jones and Mangan 1977). The pH is not always low

enough in the abomasum to cause dissociation. As the digesta empties into the duodenum CT complexes may be able to form from the free CT if the pH is above 5.5, with the action of bile secretions also preventing this, and as the digesta flows to the large intestine the gradual increase in pH to approximately 8-10 would be conducive to the CT complexes forming and then dissociating again.

2.5.1 Losses and detection of CT along the GI tract

There is evidence from the work of Perez-Maldonado (1994) and Perez-Maldonado and Norton (1996) that CT metabolites may be absorbed into the blood stream and excreted in the urine and faeces. Carbon 14 labelled studies by Terrill et al. (1994) suggest this may also be the case, although no ^{14}C was detected in the blood after feeding the sheep with ^{14}C incorporated plants, the radioisotopes were detected in the urine and liver. Terrill et al. (1994) found that in this study with the ^{14}C labelled CT, 92.5% was recovered, with 88.8% being in the digesta, 2.9% in gut tissue, 0.14% in faeces, 0.63% in urine and 0.0008% in liver. The ^{14}C label in post-abomasal gut tissue progressively increased to the end of the small intestine and then disappeared with smaller amounts detected in the proximal colon and caecum, with explanations that there was insufficient time for the label to reach the lower end of the GI tract (Terrill et al. 1994). This binding to the gut accounts for the losses in the GI tract in other studies (Terrill et al. 1994). These losses from the GI tract from these two studies are highlighted in Tables 2.5-2.7.

Table 2.5 Recovery* of 15-20 mg condensed tannin (CT) added to 15 mL digesta from rumen, abomasum, duodenum or ileum of a sheep fed on perennial ryegrass (*Lolium perenne)*/ white clover (*Trifolium repens*) pasture.

Gut segment	Recovery of CT (% of CT added)				
	Free	Protein-bound	Fibre-bound	Total	Loss/ND
Rumen	31.5	46.6	4.2	82.3	17.7
Abomasum	32.1	12.8	0.6	45.5	45.3
Duodenum	18.6	47.7	3.5	69.8	30.2
Ileum	10.3	33.1	2.3	45.7	54.3

* Corrected for background values, ND = not detectable in digesta sample, from (Terrill et al. 1994) analysed using the method from (Terrill et al. 1992b).

Table 2.6 Recovery of condensed tannin (% of total CT in feed) in free, protein-bound and fibre-bound fractions of feed, digesta from the rumen (R), abomasum (A) and ileum (I) and faeces of sheep fed *Lotus pedunculatus* containing 5.7% condensed tannin.

		Recovery of CT (% of CT in feed)				
		Free	Protein-bound	Fibre-bound	Total	Loss/ND
Feed	pH	72.0	25.4	2.6	100.0	0.0
Digesta:						
R	6.3	2.3	46.4	7.9	56.6	43.4
A	3.0	21.8	64.6	10.1	95.4	4.6
I	7.6-8.0	1.4	29.3	45.9	35.3	64.7
Faeces	-	1.1	30.9	16.2	48.1	51.9

ND = not detectable in digesta sample, adapted from Terrill et al. (1994), analysis using the method from Terrill et al. (1992b).

Table 2.7 Recovery of condensed tannin (% of total CT in feed) in free, protein-bound and fibre-bound fractions of feed, digeata from the rumen (R) and abomasum (A), urine and faeces of sheep and goats fed *Calliandra calothyrsus*/pangola grass (*Digitaria decumbens*) hay diet containing 2.25% condensed tannin.

		Recovery of CT (% of CT in feed)				
		Free	Protein-bound	Fibre-bound	Total	Loss/ND
Feed	pH	62.4	16.3	21.1	100.0	0.0
Digesta:						
R	5.6-7.2	14.4	29.8	6.3	50.5	49.5
A	2.5-3.2	15.4	30.9	6.6	52.9	47.1
Faeces		0.66	11.66	5.4	17.7	82.3
Urine					7.9	

ND = not detectable in digesta sample, adapted from Perez-Maldonado and Norton (1996), analysis using method of Perez-Maldonado (1994).

Terrill et al. (1994) reported that CT recovery from feed with added CT was close to 100%, deeming this a suitable method for analysis of feed samples, however, the recovery of CT added to digesta was much lower and even less when digesta was incubated for 4 h. This data was from one sheep only so the difference between the h of incubation may not be statistically significant. This suggests that the CT has undergone a conformational change so that is no longer able to be detected by this assay, the CT-protein complex and/or the CT-fibre complex is unable to be dissociated, or there is interference by a substance in the digesta. In a study by Perez-Maldonado and Norton (1996) which looked at the metabolism of CT in the GI tract of sheep, it was found that by using the modified *n*-butanol method of Perez-

Maldonado (1994) for determining the protein-bound fraction of CT, that more of the CT could be dissociated from the complex as compared to the method of Terrill et al. (1992b), however, results were not published on the comparison. This creates some debate as to the metabolism of CT and how the digesta is affecting the assay for CT and still if these assays are sufficient to measure all of the CT-complexes in the fractions. Carbon 14 labelled studies by Terrill et al. (1994) have shown that 92.5% of the added ^{14}C CT added to the abomasum was accounted for in the animal. Studies (Perez-Maldonado 1994, Terrill et al. 1994) analysing digesta fractions for free, protein-bound and fibre-bound CT complexes both *in vitro* and *in vivo* show apparent losses and/or undetectable amounts also, therefore, the analysis using either the methods of Perez-Maldonado (1994) or Terrill et al. (1992b) appear to have deficiencies, (Tables 2.5-2.7).

Nutritional interactions of condensed tannin complexes in the GI tract

Tannins have also been shown to have other nutritional interactions within the GI tract. These effects include enhanced by-pass proteins, anthelmintic effects, decreased DMD, increased faecal N output, inhibition of rumen microbes and digestive enzymes. (Table 2.8).

Table 2.8 Nutritional interactions with condensed tannin (CT).

Interactions	Reference
Reduction in bloat when feeding forages containing CT	McWilliam (1973), Purchas and Keogh (1984), Acharya et al. (2013), Sottie et al. (2014)
CT (2-4%) increased live weight gain, wool growth, milk yield and ovulation rate in sheep fed the legume *L.corniculatus*.	Terrill et al. (1992a), Min et al. 1999, Wang et al. (1996)
Decreased diahorrea when sheep have worm burdens	Niezen et al. (1995)
Reduced carcass fatness	Terrill et al. (1992a)
Low CT concentrations (1.5-2.0% DM) resulted in decreased protein degradation in the rumen and increased protein flow to the small intestine, i.e. enhanced "by-pass" proteins.	Barry et al. (1986), Waghorn et al. (1987)
High CT concentrations (> 6% DM) resulted in decreases in both voluntary food intake and digestibility.	Barry and Duncan (1984), Waghorn et al. (1987)
CT > 9% DM resulted in decreases in both voluntary food intake and digestibility, and also had an effect on live weight gain.	(Barry et al. (1986)
Endocrine effects found that there was a linear and positive correlation between the CT concentration and growth hormone and a negative correlation with 3,5,3'-tri-iodothyronine. Growth hormone increases nitrogen retention and lipid turnover (Muir 1983).	Barry and Manley (1986)
Decreases in population numbers of two primary fibre-degrading bacteria (*Fibrobacter succinogenes* and *Ruminococcus* spp.) when fed 30% calliandra, but did not affect the efficiency of rumen digestion	McSweeney et al. (2001)
CT from Oak decreased activity of most rumen microbe's *in vivo* and decreased RNA synthesis of these microbes. Suggested inhibition of microbial adhesion to feed.	Makkar et al. (1988)
Condensed tannin consumption can result in benefits to sheep with increased wool growth and weight gain	Barry and McNabb (1999)
Increased growth rates in goats fed feed containing CT (6.5% DM)	Moore et al.(2008)
CT binding bile acids and promoting their excretion both *in vitro* and *in vivo*	Matsumoto et al. (2011)
Reduced methane emission in cattle	Chung et al. (2013)

2.5.2 Anthelmintic effects of CT

There have been numerous studies to date reporting both the direct and indirect anthelmintic *in vivo* effects of feeding sheep and other ruminants on plants that contain CT, drenching with CT extracts (Table A2.1) and using CT extracts in *in vitro* assays. These *in vitro* assays include egg hatch assays (EHA) (Le Jambre et al. 1976, Hunt and Taylor 1989, Coles et al. 1992), larval development assays (LDA) (Taylor 1990), larval feeding inhibition assays (LFIA) (Geary et al. 1993, Alvarez-

Sanchez et al. 2005), and larval exsheathment assays (LEA) (Bauhard et al. 2006), larval migration inhibition assays (LMIA, Rabel et al. 1994, Gill et al. 1995, Barrau et al. 2005), (Table A2.2) and adult motility assays.

The *in vitro* assays are primarily used as a screening tool to identify plants that may have potential anthelmintic properties, however, may not always be effective *in vivo* due to the different physiological parameters in the gut. Different assays target different stages of the nematode life cycle and so may also speculate on the mode of action of the CT from the plant being screened. Most of these assays were developed to evaluate the efficacy of commercial anthelmintics (Jackson and Hoste, 2010).

Egg hatch assays target the eggs directly and so may disrupt the process of egg hatching (Le Jambre et al. 1976, Hunt and Taylor 1989, Coles et al. 1992). If hatching is inhibited the result will be decreasing the number of first stage larvae (L1) on pasture.

Larval feeding inhibition assays target the feeding of L1 and disrupt their feeding and so potentially kill the L1 by starvation (Geary et al. 1993, Alvarez-Sanchez et al. 2005). If feeding is inhibited and the L1 die, this will have the effect of less L1 being able to develop into infective third stage larvae (L3).

Larval development assays determine the ability of the eggs to hatch and develop through to the L3 (Taylor 1990). If inhibited then there will be less L3 to move out of the faeces to be ingested by the animal.

Larval exsheathment assays target the exsheathment of L3 (Bauhard et al. 2006). If exsheathment is delayed or inhibited death of the L3 will result or delayed exsheathment and subsequent decrease in establishment if not exsheathed before passage to next GI segment.

Larval migration inhibition assays target the motility of the L3 and its ability to move through nylon mesh (usually 20 μm), (Rabel et al. 1994, Gill et al. 1995, Barrau et al.

2005). If migration is inhibited then death of the L3 will occur because they may not be able to move out of the faeces to be ingested by the animal.

Direct effects on intestinal parasites have been demonstrated (Athanasiadou et al. 2001, Kyriazakis et al. 1996) by drenching sheep directly with quebracho extracts, however this did not have an effect on the abomasal species. Kommuru et al. (2015), however, demonstrated anthelmintic effects of reduced FEC from *H. contortus* when feeding *sericea lespedeza* to goats. The adult female worms were also found to have cuticular damage, suggesting direct effects of CT on the cuticle. However, it was demonstrated (Athanasiadou et al. 2001) in a separate experiment that the quebracho extract had anthelmintic effects on both abomasal and intestinal species in larval development viability assays.

Larval migration inhibition studies (Molan et al. 2000a) tested the L_3 larvae from the GI nematodes *T. colubriformis*, *H. contortus* and *O. circumcincta* exposed to sulla (*Hedysarum coronarium*) extract containing CT and found decreased larval migration with the *T. colubriformis* being the least affected. In another larval migration study (Molan et al. 2000b), it was found that CT extracted from *L. pedunculatus, L. corniculatus,* sulla (*Hedysarum coronarium*) and sainfoin (*Onobrychus viciifloia*) had an inhibitory effect on the migration of L1 and L3 of deer lungworm (*Dictyocaulus viviparus*) and on the L3 of deer gastrointestinal nematodes. Molan et al. (2000b) also compared the effects of CT on exsheathed and ensheathed larvae, with CT having the greater effect on the exsheathed larvae.

Larval exsheathment inhibition studies have shown CT from plant extracts of willow, tilia flowers and also red and black current leaves to have anthelmintic effects on *H. contortus* L3 from sheep or goats (Klongsiriwet et al. 2015). Quijada et al. (2015) also reported inhibition of both *H. contortus* and *T .colubriformis* L3 from goats, after exposure to 18 different plant extracts, with the most effective plants being *Vitellaria paradoxa*, *Trifolium repns, lesoedeza cuneata*, *Ribes nigrum*, *Theobroma cacao* and *Betula* spp. Williams et al. (2014a) demonstrated migration inhibition of L3 from *Oesophagostomoum dentatum* with exposure to CT rich plant extracts.

Adult nematode mortality and motility has also been shown to be affected after exposure of extracts of plants containing CT (Katiki et al. 2013; Sirama et al. 2015; Williams et al. 2014b). Katiki et al. (2013) found that adult *Caenorhabditiis elegans* motility was decreased after exposure to extracts of *Lesdedeza cuneate, Salix X sepulcralis* and *Robinia pseudoacacia*. Inhibition of worm mortility was demonstrated in adult *H. contortus* after exposure to root extracts containing CT from *Veronia amygdalina*, with up to 57% worm deaths after 24 h (Sirama et al. (2015). Motility and furthermore survival of L4 from pig *Ascaris suum* was reduced after exposure to CT extracts from a wide range of plants (Williams et al. (2014b).

Neizen et al. (1998b) demonstrated that lambs infected with *O. circumcincta* and *T. colubriformis* and fed either *L. pedunculatus* (56 g CT/kg DM) or lucerne (no CT) were found to had lower FEC when fed the Lotus. *O. circumcincta.* Worm burden and total female worm numbers was lower on the Lotus diet compared with the lucerne diet. *T. colubriformis* worm burden and male:female ratio was not affected by diet, however, the total number of eggs per female was higher in the Lotus fed sheep (Niezen et al. 1998b). This demonstrates that the CT had no effect on the intestinal worms. However, in another study feeding sulla (*Hedysarum coronarium*) which contains CT, decreased FEC and had an effect on total numbers of both abomasal and intestinal worms. The feeding of the *L. pedunculatus* and *L. corniculatus* did not affect FEC or worm burden but high levels of performance, indicated by live weight gain and wool production, were achieved on these diets (Niezen et al. 1998a). Niezen et al. (2002) conducted another study looking at the differences of feeding sulla (*Hedysarum coronarium*) compared to a non-CT plant, lucerne, and found that only abomasal worm (*O. circumcincta*) burdens and FEC were decreased and not intestinal worms (*T. colubriformis*), the reason for this being unknown.

L. pedunculatus has been shown to have an anthelmintic effect in some *in vivo* experiments (Niezen et al. 1993, Niezen et al. 1998b) but not in others (Robertson et al. 1995, Niezen et al. 1998b), however, these studies have shown live weight gains at a high rate of worm burden. No anthelmintic properties of *L. corniculatus* have been shown *in vivo* (Robertson et al. 1995, Niezen et al. 1998a). *In vitro* experiments

have demonstrated effects on eggs and larvae from both Lotus species (Molan et al. 1999, Molan et al. 2000b). This difference in anthelmintic effects may either be due to the CT concentration, or the procyanidin:prodelphinidin ratio differences in the Lotus species. *L. pedunculatus* has a higher CT concentration than *L. corniculatus* being 8% and 4%, respectively (Niezen et al. 1998a). Proanthcyanidins from *L. corniculatus* are predominantly procyanidin type subunits with molecular weight of 1990 (Foo et al. 1996) and the CT from *L. pedunculatus* are mostly prodelphinidin type subunits with MW of 2200 (Foo et al. 1997). Therefore, in the *in vivo* experiments when the plants were fed fresh feed, the CT concentrations are different but when extracted and used *in vitro* the concentrations of CT are the same. Monomers of prodelphinidin have been shown to have a greater effect of the exsheathment of infective larvae of *T.colobriformis* and *H. contortus* than monomers of procyanidin (Brunet 2006).

More recent studies using other plants species containing CT have demonstrated anthelmintic effects *in vivo* (Burke et al. 2012; Debela et al. 2012; Arroyo-Lopez et al. 2014; Werne et al, 2013; Desrues et al. 2015; Kommuru et al. 2015). Faecal egg counts were reduced in two separate studies feeding *sericea lespedeza* to goats that were infected with *H. contortus* compared to controls (Burke et al. 2012; Kommuru et al. 2015). Decreased worm burdens in lambs infected with both *H. contortus* and *T. colubriformis* were found after feeding Sainfoin (*O. viciifoliae*) but not after feeding with carob pods compared to controls (Arroyo-Lopez et al. 2014). A diet of Sainfoin resulted in a 50 % reduction in worm burden in lambs infected with abomasal *O. ostertagia* but no effect on FEC for the intestinal *Cooperia oncophora* (Desrues et al. 2015) and a reduction in FEC in lambs infected with *H. contortus* both compared to controls (Werne et al. 2013). Decreased worm burdens as well as reduced female to male ratios and eggs in utero were also reported in goats infected with *H. contortus* and fed both CT containing *Desmodium intortum* and *Sesbania sesban* (Debala et al. (2012).

There are conflicting reports on the effect of CT on abomasal and intestinal nematode species (Tables A2.1 and A2.2).

There might also be an indirect effect due to the high protein levels of the plants containing the CT. The CT released from the plant vacuoles upon mastication is free to bind to the plant protein, protecting it from degradation in the rumen therefore increasing the flow of dietary protein to the small intestine, thus making more amino acids available for absorption in the small intestine (Waghorn et al. 1987, Mueller-Harvey and McAllan 1992). Animals having a low dietary protein intake have been found to be more susceptible to disease (Coop and Holmes 1996). There is an enhanced immune response against the parasites due to increased protein availability,

2.5.3 Inhibition of digestive enzymes in the small intestine

Digestive enzymes enable the hydrolysis of the energy yielding nutrients, carbohydrates, proteins and lipids, so as breakdown products can be absorbed. The activity of an enzyme can be influenced by factors such as temperature, pH, substrate, inhibitor and activator concentrations. These factors can either enhance or inhibit an enzyme reaction. Enzyme inhibition will be driven by inhibitor and substrate concentrations and the relative affinities of the inhibitor and substrate for the active site of the enzyme.

Condensed tannin and other phenolic compounds have been shown to inhibit digestive enzymes. Lentil, pear and cocoa extracts were found to inhibit both pancreatic trypsin and α-amylase (Quesada et al. 1995). Inhibition of lipase, α-amylase and phospholipase A_2 were reported to occur in the presence of cocoa, with the types of cocoa influencing the degree of inhibition (Gu et al. 2011). *In vitro* studies have demonstrated inhibition of pancreatic α-amylase, maltase, sucrose, pancreatic lipase and pancreatic cholesterase esterase by leaf extracts of *Moringa stenopetala* containing CT (Toma et al. 2014). Horigome et al. (1988) reported that a tannin diet compared to a non-tannin basal diet fed to rats, suppressed the activity of trypsin and amylase. In contrast, this study also reported no effect of CT on lipase activity in the upper and lower GI segments, however, lipase activity was enhanced in the middle GI segment. Approximately 82% inhibition of trypsin was found to occur with exposure to sorghum CT at a concentration of 0.08 mg/mL, and approximately 95% inhibition of pancreatic α-amylase also with exposure to sorghum CT at a concentration of 0.06 mg/mL (Quesada et al. 1995). Trypsin and

pepsin have been reported to dissociate some of the protein-CT complexes in abomasal and intestinal fluids (Diaz-Hernandez et al. 1997) suggesting that it is not only pH having an effect on protein-CT dynamics but enzymes might also play a role in protein-CT complexing in the GI tract.

The optimal pH is the pH range in which an enzyme is most active, hence different enzymes are found in each GI tract segment to match the pH differences in these segments. There are also optimal temperature ranges at which enzyme reactions proceed. The rate of enzyme reactions is generally increased with increasing temperature; however, most biological enzymes are rapidly denatured above 40 ºC.

Enzyme inhibition can be classified as reversible or irreversible. Reversible inhibition can be competitive, non-competitive, un-competitive or substrate inhibition. Competitive inhibition is when the inhibitor closely resembles the chemical structure of the substrate and directly interacts with the active site of the enzyme. This inhibition is usually reversible with excess substrate displacing the inhibitor. Non-competitive inhibition occurs when the inhibitor exerts some effect on the enzyme, altering the active site so it cannot accept the substrate and can be either reversible or non-reversible. Un-competitive inhibition occurs when the inhibitor binds to the enzyme enhancing the binding of the substrate, however, is very rare. Substrate inhibition occurs at a point when there are an overwhelming number of substrate molecules that block the enzymes active site. Irreversible inhibition occurs when the inhibitor binds irreversibly to the enzyme and cannot be displaced by the substrate, for example toxic organophosphates.

Early work by McNabb et al. (1998) reported that tannin trypsin interactions in the proteolysis of BSA enhanced trypsin activity at high tannin:protein ratios, suggesting that CT may be producing conformational changes to the substrate to make it more accessible to the trypsin. Ahmed et al. (1991) demonstrated a similar phenomenon with Cockerels fed a 0-25 g/kg Salseed tannin diet (predominately HT), showing inhibition of α-amylase activity but when animals were fed a diet of 50 g/kg tannins, the amylase activity was no longer inhibited (Ahmed et al. 1991). Horigome et al. (1988) reported the activity of lipase was actually enhanced in the middle GI

segment in rats fed a tannin diet compared to a non-tannin basal diet. These examples of inhibition causing enhancement may be attributed to the occurrence of un-competitive inhibition. Gu et al. (2011) reported inhibition of pancreatic lipase, pancreatic α–amylase and phospholipase A_2 by various types of cocoa. Kinetic studies from this work revealed that a mixed type of inhibition of pancreatic lipase was occurring with cocoa procyanidin pentamer, procyanidin decamer and regular cocoa. Non-competitive inhibition was found between phospholipase A_2 and cocoa procyanidin pentamer and procyanidin decamer. However, the inhibition type was found to be competitive between the regular cocoa and phospholipase A_2 (Gu et al. 2011). In a separate study by Petersen and Hill (1991), purified tannins from sericea lespedeza were found to non-competitively inhibit cellulose enzymes. Mixed non-competitive inhibition for α-amylase and α-glucosidase has been shown *in vitro* due to extracts from Azadirachta indica (Kazeem et al. 2013).

2.5.4 Inhibition of rumen microbes

Microbial processes in the rumen enable conversion of low-quality proteins, fibrous feeds and non-protein N into nutrients for the ruminant. When a ruminant ingests a plant containing CT, masticating will release plant proteins and CT may be available to complex with these plant proteins. Tannins have also been shown to have inhibitory effects on both fibre and protein degrading rumen microbes.

Barry et al. (1986) and Waghorn et al. (1987) confirmed that ruminal degradation of protein was decreased due to CT in *Lotus pedunculatus* altering the protein or protein complexing. This in agreement with *in vitro* work by Niderkorn et al. (2012) who reported decreases in protein degradation due to the CT present in sainfoin. Makkar et al. (1988) reported inhibitory and stimulatory effects of CT from oak leaves *in vitro* and *in sacco*. Makkar et al. (1988) found decreases in the activity of urease, CMCase, glutamate dehydrogenase, in the bovine rumen, however, there was increased activity of glutamate ammonia ligase. *In vitro* studies using CT from oak leaves resulted in inhibition of the activities of urease, protease, glutamate dehydrogenase and alanine aminotransferase, however, there was an increase in the activity of glutamine synthase (Makkar et al. 1988).

Growth inhibition and CT binding to rumen microbes has been demonstrated (Nelson et al. 1997, Bento et al. 2005). A study by McSweeney et al. (2001) found that feeding 30% calliandra diet to sheep resulted in a reduction in the population of fibre-degrading rumen bacteria, *Fibrobacter succinogens* and *Ruminococcus spp*. Work by Barahona et al. (2006) is also in agreement showing that CT inhibited fibrolytic enzyme activity. The effect on bacteria appear to be quite different at low and high CT levels, with low CT levels of 0.14 mg/mL actually increasing microbial yield (Mbugua et al. 2005) and high levels of CT in the rumen have been shown to result in a lower bacterial yield (Molan et al. 2001).

2.5.5 By-pass proteins

The treatment of feeds or addition of inhibitors can be effective in protecting proteins from ruminal degradation. Some of these processes include heat treatment of feeds, formaldehyde treatment, addition of PEG and the presence of CT in the feeds. Most of the soluble proteins, including F1 leaf protein (Rubisco) which have linear structures and no cross linkages are degraded rapidly in the rumen, with almost half the N converted to ammonia. Any dietary protein that is not totally degraded in the rumen, as well as microbial proteins leaving the rumen are potentially able to be digested and absorbed in the small intestine. Dietary proteins that pass intact from the rumen to the duodenum are defined as by-pass proteins and if these are then able to be hydrolyzed by small intestinal enzymes are then referred to as digestible by-pass proteins (Kempton and Leng 1977). Two nutritional outcomes are possible from by-pass proteins. The protein is protected from ruminal degradation and digested and absorbed in the small intestine, or alternatively the protein may be excreted as faecal N.

F1 leaf protein has a high nutritional value (Mangan 1982) and if it could flow protected through the rumen this dietary protein reaching the small intestine would be advantageous to the animal. This phenomena has been demonstrated with reports of an increased flux of amino acids through the abomasum (Waghorn et al. 1987) and increased duodenal flows of non-ammonia N (Barry et al. 1986) in sheep fed a diet inclusive of CT compared to a diet treated with PEG. In the study by Waghorn et al. (1987) the flux of essential amino acids was 50% greater and the flux of non-

essential amino acids was 14% greater in the sheep on the CT diet compared with PEG treated sheep. Waghorn et al. (1987) also demonstrated that in the group fed CT there were increases in the net apparent absorption of threonine (57%), isoleucine (94%), leucine (30%), phenylalanine (93%), lysine (59%), histidine (90%), tyrosine (41%) and valine (89%), however, non-essential amino acid absorption was decreased by 10%, compared with PEG treated sheep.

If CT continued to be bound to the protein in the abomasum and also the small intestine, the protein-CT complex would pass through the GI tract and be excreted as faecal N (Perez-Maldonado and Norton 1996, Widiawati 2002, Cresswell 2007, Aufrère et al. 2013).

2.5.6 Influence of tannins on dry matter digestibility (DMD)

Dry matter digestibility is the proportion of a feed that is able to be digested by the animal or microbial enzymes, and available to be absorbed by the animal (Van Soest 1994). Dry matter digestibility has been shown to decrease when feeding a diet containing CT (Perez-Maldonado and Norton 1996, Cresswell 2007, Aufrère et al. 2013). Inhibition of digestion, causing a decrease in DMD could occur due to the complexing of CT with rumen microbes substrates (protein or fibre), or digestive enzymes in the abomasum or small intestine, and as a result may also be indicated by an increase in faecal N output (Perez-Maldonado and Norton 1996, Widiawati 2002, Cresswell 2007)**.**

Dry matter digestibility was found to be significantly lower in lambs fed a 100% calliandra diet (12 % DM CT) at 43.3% compared with lambs fed a diet of lucerne pellets (1.4 % DM CT) being 50.8% (Cresswell 2007). This is a reduction of 15 % DMD due to 100 % intake of calliandra containing 12 % DM CT. Work by Seresinhe and Iben (2003) using the rumen simulation technique also found calliandra had a DMD of 39.5- 53.5% compared to Gliricidia containing only traces of CT with a DMD of 60-65%. The percent of diet fed which equates to the CT concentration in the diet appears to determine if DMD is affected or not. There was no significant difference in DMD between a basal diet of 100% pangola grass compared to feeding sheep and goats calliandra at 300 g/kg DM with pangola grass, which is a CT

concentration of 2.25 % DM in the feed (Perez-Maldonado and Norton 1996), suggesting there is a threshold of CT concentration above which DMD is affected. In a study conducted by Aufrère et al. (2013) sheep were fed diets containing 100% sainfoin (6.4% CT), 75% sainfoin and 25% lucerne (4.6% CT), 25% sainfoin and 75% lucerne (1.4% CT) and 0% sainfoin and 100% lucerne (0% CT), it was reported that the DMD was 33.2, 46.9, 44.6 and 58%, respectively. This is a reduction in DMD of 23%, 20% and 43% for the diets containing 1.4%, 4.6% and 6.4% CT, respectively. It appears that diets from 25% to 75% sainfoin did not differ very much with respect to DM digestibility, however, a large decrease was found with the 100% sainfoin diet. In agreement with this report is results from an *in vitro* study using *Lotus corniculatus* in which it was found that DMD was not affected at CT concentrations below 8.5% DM (Miller and Ehlke 1994). This also provides evidence that there is a CT concentration threshold above which DMD is reduced.

2.6 Gastrointestinal Nematodes

2.6.1 Gastrointestinal nematodes in sheep

T. Colubriformis and *H. contortus* are two important parasitic nematodes of sheep and goats in the tropics. *H. contortus* is an important parasite in areas with well-defined summer rainfall. The infective L3 is the only stage resistant to desiccation (O'Connor et al. 2006). Infections from the pasture can only occur when eggs are passed out of the sheep via the faeces in a wet period as continual water needs to be available for the eggs to develop and survive to the infective ensheathed L3 stage. In *T. colubriformis*, in addition to the infective ensheathed L3 stage being resistant to desiccation, the egg also has an impermeable shell, which enables the eggs to survive long dry periods.

2.6.2 Life cycle of the species from the order Strongylida

Eggs are laid in the gut and undergo some cell division, on passing from the sheep with the faeces the larvae is in the eight or sixteen cell morula stage. The developing egg requires high oxygen, humidity and an optimum temperature 22 – 26 ºC. The egg develops into the L1 and at about 12 h after the egg passes from the sheep it hatches. The greatest numbers of L1 hatch 20 to 24 h after being passed in the faeces. The L1

feeds on bacteria and organic matter and then typically undergoes a moult at 44 h after the egg was passed. During this moult the complete cuticle and lining of mouth opening and excretory pore is shed. Before each moult the larvae undergoes a lethargus stage, which makes it immobile and it ceases to feed. Second moult occurs four or five days after egg passing, here the stoma closes and the larva is therefore closed off in a separate cuticle producing the L3 which is ensheathed and cannot feed until entering the host. The larvae are ingested by the host from the contaminated pasture. The L3 does not remove the second stage sheath until special stimuli (specific temperature and carbon dioxide concentration) are received from the host.

2.6.3 *Haemonchus contortus* (Barber's pole worm)

H. contortus is a haematophagus (blood sucking) "wireworm" that inhabits the abomasum of sheep, goats, cattle and other ruminants worldwide. Males (uniform red colour) are 10-20 mm long and females (red intestine with twisted white ovaries) are 18-30 mm long. Eggs pass from host to ground via the faeces where they hatch and within four days at optimal temperature (30-35°C) reach the infective ensheathed L3 stage. The larvae are ingested by the new host with grass or water. Unlike the L3, the developing egg is very susceptible to drying as the shell is permeable (Dunn 1978). Pathogenesis is due to the blood sucking of the fourth stage larvae (L4) and adult. Acute disease shows signs of anaemia, loss of wool and dark faeces. Chronic disease may only be indicated by weight loss and wool peeling in the adults. There is a substantial rise in abomasal pH (to almost 7) four days after infection and continues for about a week when the L4 are moving from the gastric pits to the surface of the mucosa (Malczewski 1971). The free-living infective larvae in the abomasum are not parasitic due to ensheathment. This parasitic phase is non-migratory and can have a prepatent period in sheep from 18-21 days. The exsheathment occurs in the rumen and is stimulated by physiologic factors, being approximately 38°C, and high concentrations of dissolved gaseous carbon dioxide. The larvae then produce an exsheathing fluid that helps the moult and also stimulates protective immunity in the abomasum. *H. contortus* L4 and adults are responsible for most of the damage in the sheep.

2.6.4 *Trichostrongylus colubriformis* (Black scour worm)

T. colubriformis are grazers (non-haematophagus) found in the duodenum of wild and domestic ruminants and occasionally pig, man and other animals. Males are 4-8 mm long and females are 5-9 mm. The eggs pass out of the host via the faeces, hatch after a day or two then develop into infective L3. Embryonated eggs (egg shell not permeable) and ensheathed L3 are highly resistant to drying (Dunn 1978). Infective larvae are ingested via food and/or water. This parasitic phase is non-migratory and can have a prepatent period from 15-23 days. Exsheathment occurs in the acid stomach where pH is then raised due to infection. Acute disease as characterised by severe enteritis, and chronic disease appears as a wasting condition. In *T. colubriformis* most of the damage is caused by the L4 and young adult stage where FEC are not high so using FEC as indication of worm burden is not very accurate (Dunn 1978).

2.6.5 Nutrition parasite interaction

Sheep almost inevitably have some level of parasitic infection. Factors including animal health, level of nutrition, and severity of infection can affect the animals' ability to cope with infection (resilience) and ability to mount an immune response that is effective against the nematode (resistance).

Apart from any nutrient competition between the parasite and host, parasites can only be harmful if they or their excretory/secretory (ES) products directly interact with the host tissues.

Subclinical infections with gastrointestinal nematodes e.g. *T. colubriformis, Teladorsagia circumcinta and H. contortus* can increase the host's protein requirement (Poppi et al. 1986, Kimambo et al. 1988, Bown et al. 1991) and severely depress appetite (Kimambo et al. 1988, Kyriazakis et al. 1996). This increased protein requirement is due to loss of endogenous N into the gut (blood, plasma, sloughed epithelial cells and mucin) which is associated with the parasite infection and the diversion of amino acids to the gut for epithelial repair and production of secretory products (Yu et al. 1998).

2.6.6 Digestive function and parasites

2.6.6.1 Morphology

Parasitic infections of the GI tract can cause alterations in the normal gut morphology, including villus atrophy, indistinct brush border, flat mucosa, as well as leaks of the vessels of the villi and lesions due to disrupted epithelial integrity (Barker 1975). Sheep infected with *Ostertagia circumcincta* exhibit changes in abomasal morphology which were mostly due to mucosal hyperplasia and also a reduction in parietal cell number (Scott et al. 1998). Jones (1982) found that lambs infected with *Trichostrongylus vitrinus* also had proximal small intestine mucosal damage and Armour et al. (1966) found mucosal sloughing by day 16 after sheep were infected with *Ostertagia circumcincta*. Apart from this mucosal damage there have been studies showing that the depth and weight of fundic mucosa were increased with infection of the abomasal parasite, *Ostertagia circumcinta* (Anderson et al. 1988), and disruption to the epithelial cell junctions have been reported in calves infected with *Ostertagia ostertagi* (Murray et al. 1970).

2.6.6.2 Gut secretions

Parasitic infections have been reported as having an effect on production and/or secretion of HCl, gastrin, pepsinogen, pancreatic and brush border enzymes. Calves infected with *Ostertagia ostertagi* were found to have a raised pH of 6.42 between 21-30 days post infection (Murray et al. 1970) and a single infection with *Teladorsaga circumcincta* in sheep resulted in raised abomasal pH of 6. Murray et al. (1970) suggested that this was due to replacement of functional parietal cells by undifferentiated cells. However, other studies using sheep infected with *Ostertagia letospicularis* also reported increased abomasal pH to 5-6 and increased gastrin levels but disagreed that it was due to cell differentiation and suggested that it is due to the influence of ES products of the parasite (Hertzberg et al. 2000) because after the anthelmintic removal of parasites, within four days the parietal cells were secreting HCl again. Another study showed similar results with adult *H. contortus* in sheep and not the larval stages inhibiting parietal cell acid production with increased abomasal pH to 5-6 (Simpson et al. 1997). These values were starting to return to normal two h post-drenching, suggesting chemical mediation for the effect on acid secretion and not parietal or enterochromaffin like (ECL) cell damage (Simpson et al.

1997). Lethal doses of *Trichostrongylus axei* in calves gave rise to abomasal pH values of 8.2 (Ross et al. 1968). When abomasal pH increases it is a normal physiological response to release gastrin. Studies reporting that gastrin levels increase with adult parasitic infection compared with uninfected controls include Simpson et al. (1997) and Fox et al. (1987), in sheep and calves, respectively. The adult worms appeared to have an effect of raising abomasal pH in the presence of increased gastrin. The effect may be on the suppression of either the parietal cell or on ECL cells, however the mechanism is as yet unknown. Gastrin also has the effect of promoting mucosal growth.

Proliferation of bacteria in raised pH of abomasal fluid, does not allow the bacteria to be lysed and utilized as a protein source. In sheep infected with *H. contortus*, abomasal pH increases has proliferation of anaerobic bacteria similar to that in the rumen 10-14 day's post-infection (Nicolls et al. 1987).

In infections with *Trichostrongylus vitrines* pancreatic enzymes, trypsin and chymotrypsin were decreased due to infection as were brush border enzyme activity of alkaline phosphatase, leucine aminopeptidase, maltase and glycyl-L-dipeptidase (Jones 1982). There have also been similar findings with *T.coluriformis* (Sykes et al. 1979).

Pepsinogen concentration appears to be elevated with infection of parasites (Armour et al. 1966, Fox et al. 1987, McKellar et al. 1990, Simpson et al. 1997). It was proposed that the pepsinogen secretion was directly stimulated by parasite ES products in infections with *Ostertagia ostertagi* and *Ostertagia circumcincta* (McKellar et al. 1990).

2.6.6.3 Other effects of gastrointestinal parasites

It has been suggested that parasite infections may not just depress feed intake but have some control over metabolic rate by altering protein metabolism and efficiency of energy utilization (Sykes and Coop 1977). It has been reported that with parasite infections, thyroxine (Prichard et al. 1974, Sykes and Coop 1977, Fox et al. 1987) and insulin levels were decreased (Fox et al. 1987).

It has been shown that infection with *T. colubriformis* resulted in changes in digesta flow and N metabolism with smaller rumen volume and larger abomasum, small intestine and caecal-colon volumes, indicating a shift of fermentation caudally (Roseby 1977). This study concluded that there was a reduction in amino acid absorption from the small intestine but a greater deamination of proteins in the large intestine with a corresponding increased absorption of ammonia that was being converted to urea and excreted. Work by Rowe et al. (1988) also showed that infection with *H. contortus* caused reduced rumen digestion and altered fermentation with increased digesta flow and changes in the pattern of volatile fatty acid production, suggesting either altered motility or some effect on microbial populations. Absorption of phosphorus from the small intestine was impaired in lambs infected with *T. colubriformis* therefore less phosphorus would be in the blood and so less would be available to be secreted in the saliva (Poppi et al. 1985).

2.6.6.4 Gut motility

It has been shown that the gut motility is altered in parasitic infections compared to uninfected controls. Lambs infected with *T. colubriformis* showed GI motility disturbances with increases in the frequency of migrating myoelectric complexes (MMC) (Gregory et al. 1985). Similar research in sheep infected with *H. contortus* showed increased antral contraction and an increase in the number of duodenal MMC per day during larvae development (Bueno et al. 1982). This study also noted an increase in duodenal digesta flow which was found to be due to ionic permeability changes in the GI mucosa. In another study, lambs infected with *T. colubriformis* had a bile over abundance in the duodenum, also suggesting motility was impaired (Poppi et al. 1985).

2.6.6.5 Tannin-parasite interaction

H. contortus ingests sheep blood and might receive a very small amount of absorbed phenolics via this route, however, the CT effect would have to be from the contact with or complexing with the cuticle (consisting mainly of proteins and lipids) and possibly from CT absorbed through the cuticle. The cuticle CT complex is pH dependant and would only occur if free CT entered the abomasum and the abomasal

pH was greater than 3 to allow the protein-CT complex to occur. Exsheathment occurs in the rumen where stable protein-CT complexes form. The effect that the CT may have on the eggs, which are permeable in this nematode, would be more probable with any free CT entering the duodenum or CT-protein complexes that are dissociated due to bile secretions. The free CT would be able to complex with the egg shells when conditions are approximately pH 3.5-7.0. As the eggs proceed down the GI tract the pH will increase. The higher pH only sees a smaller percentage of the complexes dissociated as compared with pH of < 3.0 (Jones and Mangan 1977). In infections with *H. contortus* there is a substantial rise in abomasal pH (to almost 7) about four days post infection for about a week (Malczewski 1971). At this point any free CT entering the abomasum would be in the pH range to form stable complexes with the cuticle and possibly be absorbed. Studies by Brunet et al. (2008), have shown that exsheathed L3 exposed to CT concentrations of 1200 µg/mL not penetrate the fundic explants. This was reversible when L3 were treated with polyvinylpolypyrrolidone (PVPP) and suggests that there is an interaction between the larvae and the CT in the abomasum.

T. colubriformis are duodenal grazers, therefore these nematodes would be able to ingest free, protein-bound and fibre-bound CT and if conditions were ideal, CT would be able to complex with and possibly be absorbed by the cuticle. The eggs would have the same exposure time to CT along the GI tract as *H. contortus.*

2.6.6.6 Cuticle structure

The nematode cuticle is metabolically active and selectively permeable. The cuticle covers the entire surface and the exterior openings (oesophagus and rectum), however, the cuticle lining the exterior openings has a slightly different structure to the surface cuticle. The cuticle consists mainly of protein and lipids with associated mucopolysaccharides, with collagen (different to vertebrate collagen and termed secreted collagen) being the dominant protein structure. Electron microscopy studies exposing *H. contortus* to tannin-rich plants showed structural changes to the cuticle (longitudinal and transverse folds and thicker cuticle ridges) and buccal areas by suggesting an effect on worm motility, reproduction and nutrition (Martinez-Ortiz-de-Montellano et al. 2013). Similar effects on the cuticle with shrunken and

desheveled appearance of *H. contortus* have been reported after exposure to *sericea lespedeza* (Kommuru et al. (2015). Rupture and detachment of the hypodermis in *Oesophagostomum dentum* after exposure to CT has been reported (Williams et al. 2014a). Williams et al. (2014b) also reported similar damage in Ascaris suum from pigs after exposure to CT with massage damage reported to the digestive tissue as well. Vanillin-HCl staining of worms (Cresswell 2007) showed some staining around the female vulval flap and one female was found to have some staining on the surface cuticle striae.

2.6.6.7 Egg shell

The structure of nematode egg shell is very complex. In most species of nematodes the egg shell is composed of three layers (can be 1-5 layers) secreted by the fertilised oocyte (Wharton 1980). The egg-shell consists of an outer vitelline layer, a chitin/protein complex and an inner lipid layer. The chitin micro fibrils are surrounded by a protein coat (proline being the major amino acid) to form a composite fibril (Wharton 1980) and the inner lipid layer is the main permeability barrier. The chitin/protein composite is highly resistant to environmental hazards and the mechanical and chemical resistance may be further increased by exposure to tannins (Wharton 1983).

2.6.7 Commercial anthelmintics

Anthelmintics are commonly classified according to their mode of action, spectrum of activity and effectiveness against resistant strains. The classes of broad- and narrow-spectrum anthelmintics and their mode of action that are effective against nematodes are shown in Table 2.9. There are widespread reports of resistance to these classes (Table 2.10).

Table 2.9 Broad- and narrow-spectrum anthelmintics and their mode of action.

Anthelmintic class	**Mode of action/target site**
Broad-spectrum	
Benzimidazoles (BZ, whites)	β-tubulin binding (inhibits microtubule formation, disrupts energy supply)
Imidazothiazoles (Levamisole/morantel/pyrantel, clears)	Nicotinic receptor agonist (stimulates muscle contractions leading to paralysis)
Macrocylic lactones (avermectins & milbemycins)	Glutamate-gated chloride receptor potentiators (muscle paralysis)
Aminoacetonitrile derivatives (ADD's, Monepantel)	Nicotinic receptor agonist (stimulates muscle contractions leading to paralysis)
Spiroindole (SI, derquantel)	Nicotinic receptor antagonist (blocks nerve signals, paralysis)
Narrow-spectrum	
Organophosphate compounds (OP)	Cholinesterase inhibitors (disruption of nerve transmission)
salicylanilides & substitued phenols	Proton ionophores (uncouplers of oxidative phosphorylation)
Piperazine	GABA agonist

Table 2.10 Major reported resistance to anthelmintic classes, Benzimidazoles (BZ), Imidazothiazoles (IZ), Macrocylic lactones (MLs), Aminoacetonitrile derivatives (ADD), Organophosphates (OP) in parasitic helminths.

Host	Parasite	BZ	IZ	MLs	Salicylanilides	OP	ADD
Sheep	*Haemonchus contortus*	X	Rare	X	X	X	
and/or	*Ostertagia* spp.	X	X	X	–		
goats	*Trichostronglyus* spp.	X	X	X	–		X
	Teladorsagia circumcincta						X
Pig	*Oesophagostomum* spp.	X	X	X	–		
Horse	Cyathostomes	X	X		–		
Cattle	*Cooperia* spp.	X		X	–		
	Haemonchus placei	X	X				
	Ostertagia ostertagi	X	X		–		
	Trichostronglyus axei	X			–		

Dashes indicate that the drug is not generally active against susceptible worms. Adapted from Condor and Campbell 1995, Sangster 1999 and Scott et al. 2013.

2.6.7.1 Anthelmintic resistance

Resistance is a genetically determined decline in the efficacy of an anthelmintic against a population of parasites that is generally susceptible to that drug (Sangster

and Gill 1999). The diagnostic definition given by the World Association for the Advancement of Veterinary Parasitology states that "Anthelmintic resistance is a failure to reduce FEC's by at least 95% (Coles et al. 1992). There are differences between the technical and diagnostic definitions which causes some confusion because it is now known that low levels of resistance can be present on a property and not always be detected by the FEC reduction test (Martin et al. 1989).

The first reports of anthelmintic drug resistance occurred nearly sixty years ago (Drudge et al. 1957). Resistance is now widespread and of alarming concern, particularly to the sheep and goat industries. We now have the extreme situation with resistance to all broad-spectrum anthelmintics (Table 2.12) in the tropical/sub-tropical small ruminant industry in southern Latin America (Waller et al. 1996), South Africa (Van Wyk et al. 1999), and the southern United States (Howell et al. 208).

Anthelmintic resistance has been most commonly detected in GI nematodes of ruminants, particularly sheep and goats and small strongyles of horses, but is increasingly being reported for cattle nematodes as well (Kaplan, 2004). Resistance acquired by helminths is via receptor loss or reduction in target site affinity for the drug (Köhler, 2001).

The first broad-spectrum anthelmintic, Organophosphates was introduced in 1952, followed by thiabendazole in 1961 (Brown et al. 1961) followed by, Pyrantel in 1966 and Ivermectin in 1981. Analysis of faecal samples in New Zealand by 1994 suggested 74% resistance to benzimidazole anthelmintics and 23% to levamisole-type anthelmintics (McKenna 1994). Australia now has widespread resistance to all anthelmintics including moxidectin (Love et al. 2003, Playford et al. 2014) and increasingly to Monopental (Playford et al. 2014). First reports of resistance to monopental have been made in New Zealand, in only less than four years after its release on the market (Scott et al. 2013) and also in The netherlands (Van den Brom et al. (2015). Monepantal was released Australia in 2010 and resistance has been detected in sheep and goats in 2013 (Scott et al. 2013).

2.6.7.2 Cost

The cost and availability of these commercial anthelmintics in developing countries is usually a major hurdle to their use. The use of a cheap and readily available alternative such as a fodder legume containing CT may be one alternative option worth exploring.

2.7 Conclusion

The feeding of plants containing high levels of CT have the potential to exert both anthelmintic and nutritional effects in ruminants. The CT may be complexing with the nematodes, dietary proteins, as well as rumen microbes and digestive enzymes in the small intestine.

There is a significant problem in the ruminant livestock industry with regards to resistance by GI parasites to commercial anthelmintics. There are many studies confirming that CT has a negative effect on GI nematodes. Calliandra contains a high concentration of CT and recent studies by Cresswell (2007) demonstrated that feeding calliandra inhibited egg production in sheep infected with *H. contortus* and *T. colubriformis*. It is not conclusive about the role CT plays in this anthelmintic effect but this may be clearer by determining CT availability for complexing with the worms.

A major influence on the dynamics of the interactions between the three fractions of CT, protein-bound, free and fibre-bound in the GI tract appears to be pH. Gastrointestinal tract pH possibly plays a role in determining the availability of CT to complex with the worms as well as dietary proteins, rumen microbes and digestive enzymes. If CT is producing by-pass proteins, it is beneficial but only if the CT is then released, making dietary protein available for enzyme degradation in the abomasum and small intestine. The pH at which calliandra CT complexes and dissociates needs to be investigated. There are differing opinions on the best method for analyzing CT. No direct comparison between the methods of Perez-Maldonado (1994) and Terrill et al. (1992b) have been published so further investigation is needed to clarify the best method to use for analysis of CT in plant and digesta.

CHAPTER 3 EXTRACTION AND PURIFICATION OF CONDENSED TANNIN FROM *CALLIANDRA CALOTHYRSUS*

3.1 Introduction

The tropical shrub legume *Calliandra calothyrsus* (calliandra) which contains a high concentration of condensed tannin (CT) will be used in subsequent experimental work in this thesis to investigate the *in vivo* and *in vitro* dynamics of the binding and dissociation of calliandra CT. Purified calliandra CT is required, both as the internal standard for the CT assays and as the source of pure CT for the complexing and inhibition assays.

Plants can contain a mixture of chemically distinct types of tannins which may include both CT and hydrolysable tannin (HT) (Haslam 1979). Purifying tannin is necessary if using the CT as an internal standard and also as a source of pure CT for experimental work. Hydrolysable tannin and protein concentration should also be reported to ensure the purity of the CT.

Using purified extracts from the plant of interest as an internal standard in the CT assay will give an absolute estimate of the tannin concentration being analysed and not an assay value to rate different samples. Commercially available standards e.g., tannic acid and catechin, will assay for relative amounts of tannin and must be reported as tannins per weight of the commercial standard (Hagerman and Butler 1989). The acid-butanol assay can be standardised with cyanidin, however, this would be only appropriate if the sample contained procyanidin and not for samples containing prodelphinidin or other types of CT (Hagerman and Butler 1989). Calliandra has been reported by Rosales (1999) to contain 82.6% procyanidin, 8.2% prodelphindin, 2.2% propelagonidin and 6.5% unknown anthocyanidins.

During the extraction process not all of the plant protein that is associated with the CT is removed (Hagerman and Butler 1980a), which is not surprising due to the high affinity that protein has for the CT. Hagerman and Butler (1980a) have reported a modified method of extraction with two additional steps. Initially an ethanol wash is

used to remove alcohol-soluble proteins, however, unlike cereals, legumes have insignificant amounts of this protein (Long 1961). Secondly, there is the addition of a phenol fractionation step to remove some of the remaining protein. The method reported in this chapter does not have this phenol fractionation step therefore the protein content is reported.

The aim of this experimental work is to conduct large scale extraction and purification of CT from calliandra to produce a sufficient supply of pure CT for use as the internal standard in the CT assays (Chapters 4 and 5) as well as the source of pure CT in the complexing and inhibition assays (Chapters 7, 8 and 9).

This chapter details the extraction and purification of CT from calliandra, and reports the percentage of HT and protein present in the resulting purified CT.

3.2 Materials and Methods

The current experiment was undertaken in the Nutritional Physiology and Metabolism Unit in the College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville.

3.2.1 Plants

Calliandra plants were grown in paddocks that were irrigated and regularly fertilised at the College of Public Health, Medical and Veterinary Sciences precinct at James Cook University, Townsville. The branches harvested for the extraction of CT were cut from calliandra plants that were grown in November 2002 and planted out as seedlings in March 2003, 9 months prior to harvest.

3.2.2 Laboratory analyses

3.2.2.1 Extraction of CT

Between 7.30 and 10.00 am in December 2003, branches were cut and leaves stripped from eleven calliandra trees. Approximately 10 kg of leaves were weighed into 500g batches, frozen in snap lock plastic bags and stored at -20 ºC until extraction.

Extraction of CT from calliandra was by the method reported by Broadhurst and Jones (1978) with the following modifications made by the Department of Primary Industries Agency for Food and Fibre Sciences, Animal Nutrition Institute, Health and Nutritional Biochemistry laboratory. Approximately 100-150 g batches of frozen leaves were weighed out and dry blended in a 1L glass Waring commercial laboratory blender (Waring, Connecticut, USA), after which 200-300 mL of 70 % acetone (containing 0.1% ascorbic acid) was added and blended on high for 1 min. Samples were spun for 10 min (Sigma 4K15 refrigerated centrifuge, Sigma Laborzentifugen GmbH), re-extracted twice and the diethyl ether washes were continued until clear to remove chlorophyll pigments. Samples were freeze dried (Dynavac, Dynavac Engineering Pty Ltd, Sydney) overnight and stored in glass vials desiccated at -20 ºC.

3.2.2.2 Purification of CT

Freeze dried crude CT extracts were purified in four batches on a Sephadex LH-20 (Amersham Biosciences AB, Uppsala, Sweden) column (30 x 250 mm) as shown in Plate 3.1. The amount of extract loaded onto the column for batches 1, 2, 3 and 4 were approximately 20, 30, 25 and 40 g crude CT, respectively. Crude CT dissolved in 50% methanol was loaded onto the Sephadex column which had been equilibrated with 50% methanol. The column was washed with 50% methanol to remove flavonoids and low molecular weight phenolics until A_{350nm} (Nova spec II, Amersham Pharmacia Biotech, Uppsala, Sweden) was approximately less than 0.05 absorbance units. The column was then washed with 70% acetone to remove the bound tannin fraction from the column. The eluted brown-coloured fractions each of 20-25 (also corresponding to high absorbance readings at A_{350nm}) were pooled, rotary evaporated (Heidolph VV 2000, John Morris Scientific, Chatswood, NSW) at 45 ºC, freeze dried (Dynavac, Dynavac Engineering Pty Ltd, Sydney) overnight and stored desiccated at -20 ºC in 25 mL glass vials. At completion of freeze drying, the lyophilised batches of purified CT were mixed thoroughly to produce a homogeneous product, divided into several batches and stored in glass vials desiccated at -20 ºC.

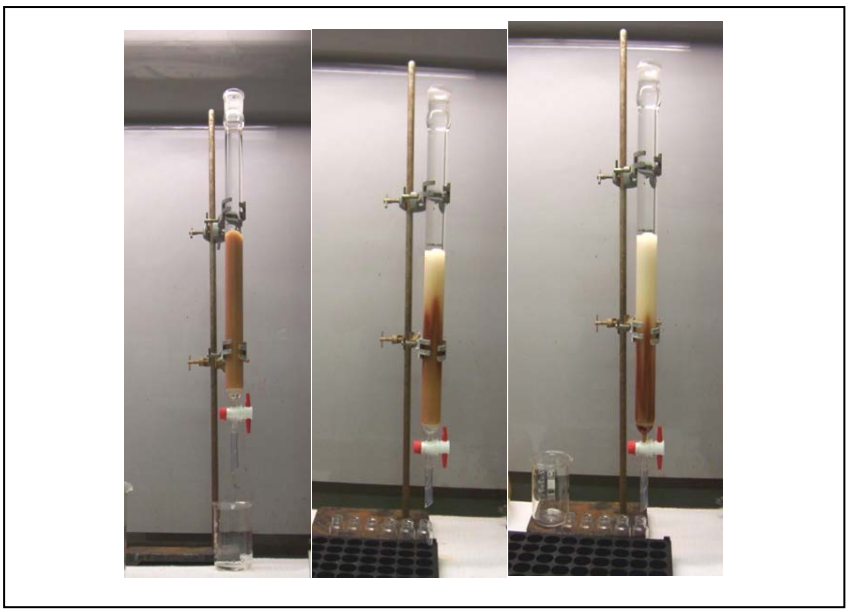


Plate 3.1 The progression of the Purification of CT extracts on a Sephadex LH-20 column.

3.2.2.3 Determination of HT

The radial diffusion assay for tannins (Hagerman 1987) was used to determine the CT:HT ratio after hydroxylaminolysis (Hagerman et al. 1997), with the following modifications. The amount of BSA added to the agarose was 0.05 g/100 mL and 3 mg of the purified CT was dissolved in 100 µL of 50% methanol. Twenty-five µL of the CT methanol solution was added to both 300 µL of hydroxylamine reagent and water (control). The water solution containing the CT was immediately loaded in 50 µL lots into the 12 x 5 mm wells of the radial diffusion plates. After incubation at 70 °C for 48 h, 50 µL of the hydroxylamine treated CT was loaded into 12 x 5mm wells. The diameter was measured twice (once and then 90 degrees to the first reading) across the diameter of all assays.

3.2.2.4 Determination of protein contamination

Bradford Assay (Bradford 1976) was used to determine the protein content of the sample as µg of protein/mg of sample. The assay was calibrated with BSA concentrations from 0-10µg/mL. Bradfords reagent and BSA micro standards (1 mg/mL) were purchased from Sigma-Aldrich, Australia.

3.3 Results

3.3.1 Extraction and purification of CT

The Sephadex LH-20 elution profile and the extraction and purification yields for CT are shown in Figure 3.1 and Table 3.1, respectively.

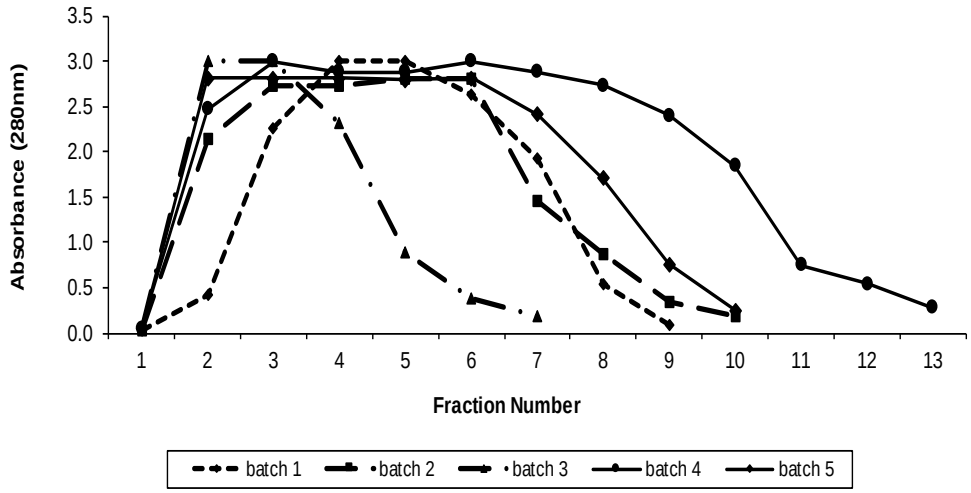


Figure 3.1 Sephadex LH-20 elution profiles of tannin extracts from batches 1-4.

Table 3.1 Condensed tannin (CT) yields during purification of calliandra.

Purification step	Product	Extracted Wt. (g DM)	Yield (%)
	Frozen plant	890.51	100.00
Solvent extraction	Crude CT	116.93	13.13
Sephadex LH-20	Pure CT	30.29	3.40

3.3.2 Hydrolysable tannin concentration

Results from the radial diffusion assays showed that the purified CT contained 95 and 5 % CT and HT, respectively.

3.2.3 Protein concentration

Traces of protein were detected in the purified CT extracted from calliandra which was not able to be removed during extraction and purification. This was at a concentration of 15.7 µg protein per mg of pure CT, which is 1.6% (by weight).

3.4 Discussion

Large scale extraction and purification of CT from calliandra was successful with minimal contamination of protein and HT. The process of extraction and purification of CT from plant material required approximately 5 months laboratory work to obtain the 12.67 g DM of pure CT which is a 3.4% yield from 890.51 g DM of calliandra leaves. Pure CT yields of approximately 7% have been reported for pinto bean hulls and 15% from sorghum (Hagerman and Butler 1980a).

The protein content of the purified CT was determined to be 1.6% (by weight) using the modified method of Broadhurst and Jones (1978) for the extraction of CT. The modified method of Hagerman and Butler (1980a) involving two extra steps to remove more protein, reported contamination of not greater than 2-3% (by weight) for sorghum tannin.

3.5 Conclusion

It can be concluded that the purified CT with the HT and protein contamination being only 5 % and 1.6 %, respectively, can be confidently used as a suitable internal standard for the CT assays in Chapters 4, 5 and 7 and to be utilised as the source of pure CT in the protein-CT complexing, inhibition of digestive enzymes and other *in vitro* assays in chapters 7, 8 and 9.

CHAPTER 4 COMPARISON OF THE METHODS OF TERRILL ET AL. (1992B) AND PEREZ-MALDONADO (1994) FOR THE ANALYSIS OF THE THREE FRACTIONS OF CONDENSED TANNIN (FREE, PROTEIN-BOUND AND FIBRE-BOUND) IN PLANTS.

4.1 Introduction

Purified CT extracted from the tropical shrub legume calliandra will be used in CT assays as the internal standard and as the source of pure CT in *in vitro* complexing, digestive enzyme inhibition and other *in vitro* assays. Calliandra is the feed source in experimental work for subsequent digesta analysis. Chapter 3 showed that the extraction and purification of CT from calliandra was possible with minimal contamination from protein (1.6%) and HT (5.5%), deeming this CT suitable for subsequent use.

Tannins are naturally occurring water-soluble plant polyphenols (secondary plant metabolites), which have the ability to form complexes with proteins, minerals and carbohydrates, including cellulose. Condensed tannin exists in plants as protein-bound, fibre-bound and unbound (free) fractions.

These three plant fractions are currently assayed using the modified method of Terrill et al. (1992b), based on the original *n*-butanol-HCl method of Porter et al. (1986), which incorporates an acetone-water-diethyl ether extraction and SDS extraction. This method has since been modified by Perez-Maldonado (1994), for analysis of digesta and faecal samples, as the Terrill et al (1992b) method did not sufficiently dissociate the CT from the protein. However, there still appears to be limitations of this method. For assay of the protein-bound fraction, the pH has been changed from 8.0 to 10.0 by replacing the tris-HCl buffer (HCl was claimed to interfere with the assay) with tri-ethanolamine, as well as using a substantially lower concentration of 2-mercaptoethanol, 1% instead of 5%, to make the procedure safer in the laboratory. The fibre-bound tannin assay was determined in methanol instead of water and a final extractant of ethyl acetate was added to the free CT estimation to eliminate

small phenolics. However, a clear comparison between the two methods has not been reported.

The experimental work in chapters 5 and 7 require that CT fractions from both plant material and digesta be analysed. Therefore, it is first necessary to compare the CT assays of Terrill et al. (1992b) and Perez-Maldonado (1994) to determine which of the two methods is best suited for routine analysis of both plant material and digesta samples. Any necessary modifications will be made to the method before the samples from subsequent experiments involving plant feeds, digesta, faeces and *in vitro* complexing studies are analysed. Appropriate assay blanks for plants and digesta will also be investigated.

It was hypothesised that:

i. *analysis of plant and digesta samples for the three fractions of CT as well as the calculated total CT by the methods of Perez-Maldonado (1994) and Terrill et al. (1992b) will give the same concentrations of CT.*

The current experimental work was undertaken to examine this hypothesis. A comparison of the methods of Terrill et al. (1992b) and Perez-Maldonado (1994) will be undertaken using the plants calliandra and desmanthus and using rumen digesta from calliandra fed lambs. Each step of the analysis for each method will be compared.

4.2 Materials and Methods

The current experiment was undertaken in the Nutritional Physiology and Metabolism Unit in the College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville. Collection of the rumen digesta was conducted with Experimental Ethics Approval No A826_03 and in accordance with the *Guidelines for Housing and Care of Laboratory Animals* issued by The Experimental Ethics Review Committee of James Cook University.

4.2.1 Samples and sampling

4.2.1.1 Calliandra

Two branches were cut at 1m above the ground from a calliandra shrub that had approximately 7-7.5 month's regrowth. The branches were 2.5-3 m long with a diameter of 30-40 mm. All leaves from the branches were removed to get an average CT concentration from the shrub legume. Leaves were gently stripped in small amounts and immediately put into plastic snap lock bags between two layers of dry ice. These were left for half an hour and then transferred to the -80 ºC freezer. The following day these samples were freeze dried (Dynavac, Dynavac Engineering Pty Ltd, Sydney) for 24 h, ground using a hammermill (Dietz-motoren KG, Retsch GmbH, West-Germany) to pass through a 1 mm screen and stored desiccated at room temperature pending analysis for CT. An extra 200 g of leaves were cut for DM analysis.

4.2.1.2 Desmanthus

Desmanthus plants that were 1m tall with approximately 2.5-3 months regrowth were harvested and treated as above for calliandra with whole plants being used. The paddock in which desmanthus were sampled from was a mixture of desmanthus species. In addition separate samples of leaves and stems only were collected and processed for reporting of CT concentration. Leaves only were used for the comparison of methods in this chapter.

4.2.1.3 Rumen digesta

Rumen digesta was obtained from lambs that had been previously been fed a diet of either 100% fresh calliandra or 100% lucerne pellets (Lockyer lucerne, Gatton, Queensland). The rumen digesta samples were obtained during the experimental period for the experiment outlined in Chapter 5. Immediately after the lambs were humanely slaughtered with a captive bolt, the digestive tracts were tied off and removed as quickly as possible. The contents of the individual rumens were emptied into a beaker, mixed thoroughly, and a 15 mL sub-sample immediately placed on ice and subsequently stored at -20 ºC until analysis. The vials were place in a -80 ºC freezer for a minimum of 3 h and then freeze dried (Dynavac, Dynavac Engineering Pty, Ltd, Sydney) for approximately 31 h.

4.2.2 Laboratory analysis

4.2.2.1 Method of Perez-Maldonado (1994)

Extraction and analysis was carried out as per Perez-Maldonado (1994) with five replicates per sample. In this method Perez-Maldonado does not make mention of blanking. In the published method Perez-Maldonado and Norton (1996) it reported that the fibre-bound CT is analysed using the standards in a methanol base.

4.2.2.1 Method of Terrill et al. (1992b)

Extraction and analysis of samples was carried out as described by Terrill et al. (1992b) with the following modifications also using five replicates per sample.

4.2.2.2 Modified extraction procedure

During extraction sample size and extraction volumes were one-third of that used by Terrill et al. (1992b), 16 mL glass screw cap vials were used instead of 50 mL poly-allomer centrifuge tubes (Falcon tubes), with all samples being spun at 3000 rpm (Sigma 4K15 refrigerated centrifuge, Sigma Laborzentifugen GmbH) instead of 27 000 g due to the unavailability of a higher speed centrifuge at the time of analysis. For the free and protein-bound CT the supernatant was made up to a total of 25 mL instead of 100 mL.

4.2.2.3 Modified analysis procedure

For the free and protein-bound CT fractions the assays were carried out using 0.5 mL sample and 5 mL *n*-butanol-HCl solution. For plants with a high CT concentration, 50 µL of sample plus 450 µL distilled water and 5 mL *n*-butanol-HCl solution was used in the assay. For the fibre-bound CT fraction 10 mL *n*-butanol-HCl solution plus 1 mL SDS solution was added to the residue and the centrifugation was again carried out at 3000 rpm. Absorbance at 550nm was measured using a Novaspec II, spectrophotometer (Amersham Pharmacia Biotech, Uppsala, Sweden).
Modified blanking procedure.

In the method of Terrill et al. (1992b) all samples were blanked with an extract from non-tannin containing plant or digesta samples as determined in section 4.3.6, instead of the addition of the CT to plant extracts. To achieve a set of standards, you would

then need to add differing amounts of stock therefore adding a different dilution factor to the set and now creating a different background of colour and impurities. In this work the analysis of different plants and digesta samples (seven different regions of the GI tract) are being analysed simultaneously so it was decided to use traditional blanks instead of adding impurities directly to the standards. No mention of blanking procedure is made by Perez-Maldonado (1994).

4.2.2.4 Standards

Internal standards were made from purified CT extracted from calliandra as described in chapter 3. Details of standards used for the three fractions of CT are presented in Table 4.1 below.

Table 4.1 Condensed tannin (CT) stock concentration, concentration range of standards and diluents used for the standards in assays for free, protein-bound and fibre-bound condensed tannin.

	Free CT	**Protein-bound CT**	**Fibre-bound CT**
Stock (mg/mL)	6	6	6
Diluent – Modified Terrill	Deionised water	SDS solution	SDS solution
Diluent – Perez-Maldonado	Deionised water	SDS solution	methanol
Range (mg/mL)	0.00 – 6.0	0.00 – 6.0	0.00 – 6.0

Initially the assay blanks used were the 0.00 g/mL standards. Details of blanks subsequently used are outlined in section 4.3.6.

4.3 Statistical Analysis

Raw data were initially tabulated in Microsoft Office Excel 2003 and treatment effects analysed in SPSS 14.0 for Windows (SPSS Inc., Chicargo, Illinois, USA). Data on comparisons between the two methods described for analysis of CT fractions using calliandra and desmanthus as well as data for free CT comparing mixing wheel and vortexing steps were subjected to one way analysis of variance (ANOVA). Data for protein-bound CT analysis comparing drying samples in an oven and no drying steps were subjected to Kruskall Wallace k sample test. Where Kruskell Wallace k test found significant effects, the mean values were subjected to a Mann-Whitney

test. Comparison of multiple steps with the two methods described for CT fractions were subjected to Multivariate analysis of variance (MANOVA). Comparisons between the two methods using rumen digesta were analyzed using an independent t-test. Effects were considered significant at a probability value of $P < 0.05$.

4.4 Results

4.4.1 Direct Comparison of the two methods using calliandra and desmanthus.

Data on free, protein-bound, fibre-bound and total CT concentration in calliandra and desmanthus using the modified method of Terrill et al. (1992b) and the method of Perez-Maldonado (1994) are presented in Table 4.2.

Table 4.2 Concentration [% dry matter (DM)] of free, protein-bound, fibre-bound and total condensed tannin (CT) in calliandra and desmanthus using the modified method of Terrill et al. (1992b) and the method of Perez-Maldonado (1994).

		Condensed tannin concentration (% DM)			
		Free CT	**Protein-bound CT**	**Fibre-bound CT**	**Total CT**
Plant	**Method**				
calliandra	Modified Terrill	7.95 ± 0.42	2.33a ± 0.05	0.36a ± 0.04	10.64 ± 0.41
calliandra	Perez-Maldonado	8.69 ± 0.93	1.01b ± 0.09	1.01b ± 0.09	10.68 ± 0.90
P value		0.493	0.000	0.000	0.973
desmanthus	Modified Terrill	28.18a ± 1.46	4.34a ± 0.04	0.49a ± 0.03	33.01 ± 1.47
desmanthus	Perez-Maldonado	33.91b ± 1.06	0.72b ± 0.05	1.00b ± 0.05	35.63 ± 1.03
P value		0.013	0.000	0.000	0.183

Values within a column with different following letters differ significantly for the same plant ($P < 0.05$).

There were significant differences in the concentration of CT between the two methods for free, protein-bound and fibre-bound fractions for desmanthus and significant differences in the concentration of CT between the two methods for the protein-bound and fibre-bound CT fractions for calliandra. There were no significant differences in the total CT concentration for either method or plant analysed. Given no significant differences in total CT concentration, it was reasonable to suggest that looking at each step in the two methods might give insight into the differences in CT concentration for each fraction. Although there were significant differences in all three fractions of CT using desmanthus and only differences

between two of the fractions using calliandra, it was decided to proceed using calliandra samples only for the remainder of the comparisons. Calliandra was used in the subsequent chapters of experimental work and the purified CT being used for the internal standards was extracted from calliandra. The protein-bound CT fraction has attracted the most controversy, with respect to the two methods and there was a significant difference with calliandra.

4.4.2 Extraction and analysis of free CT

4.4.2.1 Comparison of the extraction methods for free-CT in calliandra using the methods of Perez-Maldonado (1994) and the modified method of Terrill et al. (1992b).

Data on free CT concentrations in calliandra that were extracted using the modified method of Terrill et al. (1992b) and the method of Perez-Maldonado (1994), as well as the modified method of Perez-Maldonado (1994) that has replaced the mixing wheel with vortexing are presented in Table 4.3.

Table 4.3 Concentration [% dry matter (DM)] of free condensed tannin (CT) in calliandra using the modified method of Terrill et al. (1992b) and Perez-Maldonado (1994) with modification to compare extraction protocols of vortex versus mixing wheel.

Method and protocol	Free CT concentration (%DM)
Modified Perez-Maldonado (vortex)	7.63 ± 0.78a
Perez-Maldonado (mixing wheel)	9.13 ± 0.52a
Modified Terrill (vortex)	7.95 ± 0.42a
P value	0.172

Values within a column with different following letters differ significantly ($P < 0.05$).

Using the mixing wheel during extraction of free CT did produce a slightly higher value than extracting with vortexing, however this was not significant ($P = 0.172$).

4.4.3 Extraction and analysis of protein-bound CT

4.4.3.1 Comparison of oven drying versus no oven drying for the extraction of protein-bound CT in calliandra using the method of Perez-Maldonado (1994) and the modified method of Terrill et al. (1992b).

Data on concentration of protein-bound CT in calliandra that had been subjected to oven drying using the method of Perez-Maldonado (1994) or no oven drying using the modified method of Terrill et al. (1992b) and the method of Perez-Maldonado (1994) which has been modified to delete the oven drying step, and after extraction of free CT using the modified method of Terrill et al. (1992b) are presented in Table 4.4.

Table 4.4 Concentration [% dry matter (DM)] of protein-bound condensed tannin (CT) in calliandra using the modified method of Terrill et al. (1992b) and Perez-Maldonado (1994) with modification to compare extraction protocols.

Method	**Protein-bound CT concentration (%DM)**
Modified Perez-Maldonado (no drying)	1.52 ± 0.19a
Perez-Maldonado (drying)	0.93 ± 0.06a
Modified Terrill (no drying)	2.33 ± 0.05b

Values within a column with different letters differ significantly ($P < 0.05$).

The oven drying step produced a lower value for protein-bound CT compared with the other methods with no oven drying. The results for the protein-bound CT analysed for the method of Perez-Maldonado (1994) with drying and no drying were not significantly different ($P = 0.05$). There were significant differences ($P = 0.002$) between the concentrations of protein-bound CT for the two methods that did not oven dry the samples and also between drying versus no drying for samples analysed with the Perez-Maldonado (1994) method and the modified Terrill et al. (1992b) method ($P = 0.009$). When samples were not subjected to the drying step, the concentration of protein-bound CT was significantly higher, the highest value being obtained using the modified Terrill et al. (1992b) method. It was decided to continue the comparisons, hence without oven drying, with the modified method of Terrill et al. (1992b).

4.4.3.2 Comparison of the different SDS solutions used for the extraction of protein-bound CT in calliandra using the methods of Perez-Maldonado (1994) and the modified method of Terrill et al. (1992b).

Data on concentration of protein-bound CT using the different SDS extraction solutions for the two methods being compared are presented in Table 4.5. The

concentration of protein-bound CT was significantly higher ($P < 0.001$) when extracted with the SDS solution for the modified Terrill et al. (1992b) method compared with the concentration of protein-bound CT extracted with the SDS solution for the Perez-Maldonado (1994) method.

4.4.3.3 Comparison of the number and length of SDS extractions for the determination of protein-bound CT in calliandra for the methods of Perez-Maldonado (1994) and the modified method of Terrill et al. (1992b).
Data on concentration of protein-bound CT using a different number of SDS extractions and different lengths of time for extractions for the two methods being compared are presented in Table 4.5. Irrespective of the SDS solution, the samples that were SDS extracted 2 x 45 min for protein-bound CT had significantly higher concentrations of protein-bound CT ($P = 0.000$).

4.4.4 Analysis of fibre-bound CT

4.4.4.1 Comparison of the successive 80% methanol and butanol washes versus no wash used for the determination of fibre-bound CT in calliandra for the methods of Perez-Maldonado (1994) and the modified method of Terrill et al. (1992b).
Data on concentration of fibre-bound CT after treatment with or without a methanol wash for the two methods being compared are presented in Table 4.5. Irrespective of the SDS solution and the number and length of time of SDS extractions the samples that were treated with the methanol wash had significantly higher fibre-bound CT concentrations ($P < 0.001$, 0.001 and 0.006, Table 4.5).

Table 4.5 Concentration [% dry matter (DM)] of free, protein-bound, fibre-bound and total condensed tannin (CT) in calliandra for the modified method of Terrill et al. (1992b) and Perez-Maldonado (1994) with modification to compare extraction mixtures, extraction times and wash versus no wash in the fibre-bound analysis.

CT		**Condensed tannin concentration in calliandra (% DM)**							
Free	**Vortex (n = 40)**	13.97 ± 0.33 [Modified Terrill et al. (1992b)]							
		Perez-Maldonado SDS				**Modified Terrill SDS**			
Protein-bound	**Extraction times**	2 x 45min		**1 x 75min**		**2 x 45min**		1 x 75min	
	(n = 10)	1.16a ± 0.03		**0.82b ± 0.03**		**1.42c ± 0.03**		0.94d ± 0.03	
	P value	0.000				0.000			
Fibre-bound		washes	no wash	**washes**	no wash	washes	**no wash**	washes	no wash
	(n = 5)	1.97 ± 0.13	1.24b ± 0.11	**2.09 ± 0.16**	1.59b ± 0.11	2.28 ± 0.05	**0.63 ± 0.04**	2.43 ± 0.17	1.11 ± 0.07
	P value	0.000		0.005		0.000		0.000	
Total	**(n = 5)**	18.43a ± 0.89	17.86a,c ± 0.99	**16.98a,d ± 0.89**	15.33b,c,d,e,f,h ± 0.64	17.13a,e ± 0.89	**16.59a,f ± 0.74**	17.35a,g ± 0.89	16.08a,h ± 0.89
	P value	0.001		0.006		0.000		0.000	

Values within rows with different letters differ significantly ($P < 0.05$). Shading denotes unmodified pathway for each method.

4.4.5 Comparison of the methods of Terrill et al. (1992b) and Perez-Maldonado (1994) using rumen digesta

Data on the concentration of free, protein-bound, fibre-bound and total CT in rumen digesta from sheep previously fed calliandra and analysed using the modified method of Terrill et al. (1992b) and Perez-Maldonado (1994) are presented in Table 4.6.

Table 4.6 Concentration [% dry matter (DM)] of free, protein-bound, fibre-bound and total condensed tannin (CT) in rumen digesta from sheep previously fed calliandra using the methods of Terrill et al. (1992b) and Perez-Maldonado (1994).

CT	Concentration of CT (% DM)		
Free	1.31 ± 0.05 [Modified Terrill et al. (1992)]		
Protein-bound		Perez-Maldonado	Modified Terrill
	(n = 5)	2.50a ± 0.06	3.18b ± 0.02
	P value	0.000	
Fibre-bound	(n = 5)	1.70a ± 0.13	0.77b ± 0.03
	P value	0.004	
Total	(n = 5)	5.84a ± 0.03	5.50b ± 0.02
	P value	0.003	

Values within rows with different letters differ significantly ($P < 0.05$).

Protein-bound CT concentrations were significantly higher ($P < 0.001$) in the samples analysed by the modified method of Terrill et al. (1992b) compared with the protein-bound CT concentrations in the samples analysed by the method of Perez-Maldonado (1994). Fibre-bound and total CT were significantly higher in samples analysed by the method of Perez-Maldonado (1994) compared with the fibre-bound and total CT samples analysed by the method of Terrill et al. (1992b) with P values of 0.004 and 0.003, respectively.

4.4.6 Determination of suitable blanks

Data on the concentration of free, protein-bound, fibre-bound and total CT in various locally available plants as well as lucerne pellets and rumen digesta from lambs previously fed calliandra or lucerne pellets and analysed using the modified method of Terrill et al. (1992b) are presented in Table 4.7.

Table 4.7 Condensed tannin (CT) concentration as % dry matter (DM) in *calliandra calothyrsus, Albizia lebbeck, Sesbania grandiflora, desmanthus virgatus*, lucerne pellets and rumen digesta from lucerne and calliandra fed lambs (n = 5).

	Condensed Tannin (% DM)			
Plant or digesta	**Free**	**Protein-bound**	**Fibre - bound**	**Total**
Calliandra calothyrsus	9.84	1.21	0.95	12.00
Albizia lebbeck	0.33	0.01	0.01	0.35
Sesbania grandiflora	4.08	0.77	0.86	5.71
Desmanthus virgatus	37.14	4.38	1.22	42.74
lucerne pellets	0.48	0.74	0.18	1.40
Rumen Digesta (lucerne fed lambs)	0.35	0.20	0.01	0.56
Rumen Digesta (calliandra fed lambs)	1.78	3.44	1.30	6.52

The plant that had the lowest concentration of total CT(0.35 % DM) was *Albizia lebbeck,* and the digesta from the lucerne fed lambs was the lowest in total CT concentration at 0.56% DM.

4.5 Discussion

The total concentrations of CT from the analysis of calliandra and desmanthus by the two CT methods being compared were not significantly different. The total CT values obtained for calliandra was almost identical for the two different methods (Table 4.2). There were, however, significant differences in the concentrations of all fractions of CT in desmanthus, and significant differences in the protein and fibre-bound CT fractions in calliandra.

If CT analysis was being performed for the purpose of reporting the total CT concentration only, then either method would suffice. The modified method of Terrill et al (1992b) is less complicated and has fewer steps, but for safety reasons the method of Perez-Maldonado (1994) utilises a substantially lower concentration of highly toxic mercaptoethanol in the SDS protein-bound CT extraction mixture.

4.5.1 Direct Comparison of the two methods using calliandra and desmanthus.

There were no significant differences in total CT concentration when samples were analysed using the methods of Perez-Maldonado (1994) and the modified method of

Terrill et al. (1992b),(Table 4.2, total CT concentrations of 10.64 and 10.68 % DM) and (Table 4.5, 16.98 and 16.59 % DM). It is therefore reasonable to suggest that by comparing analysis at individual steps in the methods, this might identify the factors causing the differences in the concentrations of the fractions of CT. This will enable some modifications to the protocols for the best outcome of analysis. With respect to the free CT, the concentration tended to be slightly higher for the samples analysed by the method of Perez-Maldonado (1994), with this only being significant for the desmanthus ($P = 0.013$) and not for the calliandra ($P = 0.493$). The reason that these two plant samples behaved differently may be the fact that the CT internal standard was extracted and purified from calliandra which is a different plant to desmanthus. These plants may have differing procyanidin contents. There are differing reports in the literature to suggest that this may or may not be the case. Procyanidin content of the CT seems to affect the CT assay, with a higher procyanidin content yielding a higher absorbance in the assay for the same tannin concentration (Kraus et al. 2003). This is in contrast to reports that there are no differences in the yield due to procyanidin and prodelphinidin composition of the extract (Porter et al. 1986). Free CT from both plants were analysed at 557 nm and 558 nm (CT assay is read at 550 nm) to give an indication of the cyanidin and delphinidin ratios, respectively. Calliandra gave similar absorbance reading at both wavelengths, with the difference being 0.0008 absorbance units. The procyanidin:prodelphindin:profistedinidin:propelargonidin has been reported by Rosales (1999) for a variety of tropical forage legumes. Rosales (1999) reports that 82.6% of CT in calliandra is procyanidin, 8.7% is prodelphinidin and only 2.2% is made up of propelargonidin. For desmanthus the difference between the absorbance readings at the different wavelengths were slightly greater at 0.0074 and 0.002 absorbance units for desmanthus leaves and stems respectively.

There were significant differences between the concentration of the protein and fibre-bound CT fractions using the two different methods for both calliandra and desmanthus (Table 4.2). There are obviously differences in the two methods for the analysis of the protein-bound CT as reported in the literature (Perez-Maldonado 1994). Results for concentration of protein-bound CT are consistently higher in samples analysed by the modified method of Terrill et al (1992b), being 2.3 times

and 6 times greater in samples extracted from calliandra and desmanthus, respectively, compared with those analysed by the method of Perez-Maldonado (1994). The opposite trend is seen for concentration of fibre-bound CT, which are consistently lower in samples analysed by the modified method of Terrill et al (1992b), being 2 times and 2.8 times lower in samples extracted from calliandra and desmanthus, respectively, compared with those analysed by the method of Perez-Maldonado (1994). This confirms that the modified method of Terrill et al (1992b) is more effective at dissociating the protein from the protein-CT complex than the method of Perez-Maldonado (1994). Consequently, protein that is not being dissociated by the Perez-Maldonado (1994) method appears to be now incorporated as fibre-bound CT in the following step for the analysis of fibre-bound CT. No published reports have been found comparing these two methods directly, however, Perez-Maldonado (1994) modified the original method of Terrill et al. (1992b) after suggestions were made by Terrill et al. (1994) that the method was not effective at releasing protein from the protein-bound CT. Subsequent studies by Perez-Maldonado and Norton (1996) using digesta samples claims that the method of Perez-Maldonado (1994) is more effective at releasing the protein from the protein-CT complex than the method of Terrill et al. (1992b), however, no results for this have been published.

It was decided to proceed with the comparison of protocols using calliandra samples only. The reasons being that calliandra is the plant being used in subsequent chapters of experimental work with lamb digesta and *in vitro* complexing studies, and the CT internal standards in the CT assay have been extracted and purified from calliandra. The two methods being compared in this chapter demonstrate that the protein-bound CT fraction for analysis is the most controversial of the fractions with respect to the two methods especially for analysing digesta samples.

4.5.2 Extraction and analysis of free CT

4.5.2.1 Comparison of the extraction methods for free-CT in calliandra using the methods of Perez-Maldonado (1994) and the modified method of Terrill et al. (1992b).

The method of Perez-Maldonado (1994) uses a mixing wheel to mix the samples instead of vortexing the samples during the extraction of free CT. A comparison was

made between mixing and vortexing within the protocol of Perez-Maldonado (1994) to determine whether this affected the concentration free CT extracted from the samples. These results were also compared with the free CT extraction procedure of the modified method of Terrill et al. (1992b) which uses vortexing to mix the samples for extraction. Using the mixing wheel for mixing the samples during extraction of free CT did result in a slightly higher value than extracting by vortexing as seen in Table 4.3 and also the original comparison in Table 4.2, however, this was not significant ($p = 0.172$). Vortexing is faster and easier than using the mixing wheel therefore the subsequent mixing procedure during extraction for determination of free CT concentration was with a vortex. The literature cites the protein-bound CT fraction is where the major differences are in the two methods.

No comparisons were made here between the extraction mixes to determine whether if there is a difference in free CT concentration using a separate or combined diethyl ether wash to remove pigments after either mixing or vortexing with both methods. There is evidence, however, that there were no significant differences in free CT concentrations with each original protocol. The modified method of Terrill et al. (1992b) uses a combined acetone-water, diethyl ether mix whereas Perez-Maldonado (1994) uses a separate diethyl ether wash with the acetone-water extraction mix. The fastest way to extract is with the combined acetone diethyl ether wash. With samples that contain a lot of pigment e.g. calliandra compared with digesta samples an extra diethyl ether wash may prove necessary. This is still faster, and reduces the steps from three or more pigment washes with the Perez-Maldonado (1994) method to one or two wash with the modified Terrill et al. (1992b) method.

The results in sections 4.3.2 and 4.3.3 are favourable with the extraction and mixture and method of extraction of free CT using the modified method of Terrill et al. (1992b). Subsequent comparisons between the two protocols for extraction and analysis of protein and fibre-bound CT extracted and analyse free CT by the modified method of Terrill et al. (1992b).

4.5.3 Extraction and analysis of protein-bound CT

4.5.3.1 Comparison of oven drying versus no oven drying used for the extraction of protein-bound CT in calliandra using the method of Perez-Maldonado (1994) and the modified method of Terrill et al. (1992b).

Perez-Maldonado (1994) added the oven drying of the samples before the extraction of protein-bound CT. This oven drying step is presumably to evaporate the acetone from the samples before the addition of the SDS protein extraction mix. The concentration of protein-bound CT was the lowest from the samples analysed with the method of Perez-Maldonado (1994), which included the oven drying step, compared with the analysis by the modified Terrill et al. (1992b) protocol which does not contain an oven drying step (Table 4.4). The protein-bound CT concentration, as also seen in Table 4.2, was highest with the modified method of Terrill et al. (1992b) compared with the methods of Perez-Maldondao (1994) with and without oven drying the samples. This again highlights the fact that the modified method of Terrill et al. (1992b) was more efficient at dissociating the CT from the protein compared with the method of Perez-Maldonado (1994). This was a comparison between plant samples and not digesta as to where it is claimed (Terrill et al. 1994, Perez-Maldonado and Norton 1996) that the problem is with dissociation of CT from protein. Analysis was also made to compare the methods from samples analysed with rumen digesta from calliandra fed lambs (Table 4.6). Protein-bound CT concentration in rumen digesta samples analysed by the two methods being compared was significantly higher in the samples analysed by the modified method of Terrill et al. (1992b), as has been consistently found in the plant samples analysed. This reinforces the argument for the use of the method of Terrill et al. (1992b), for the analysis of protein-bound CT fraction for both plant and digesta samples.

4.5.3.2 Comparison of the different SDS solutions used for the extraction of protein-bound CT in calliandra using the methods of Perez-Maldonado (1994) and the modified method of Terrill et al. (1992b).

The SDS protein-bound CT extraction mix used by Terrill et al. (1992b) has a high concentration of 2-mercaptoethanol at 5% and a pH of 8, whereas the SDS extraction mix of Perez-Maldonado (1994) has a concentration of 2-mercaptoethanol at 1% and a pH of 10. After analysing a considerable number of samples for CT it may be a

better option to use a safer concentration of mercaptoethanol in the extraction mix than both methods employ. The pH used in the SDS solution of Perez-Maldonado (1994) was 10, but it is very doubtful that this pH is found physiologically in the GI tract of an animal where the complexing and dissociation of CT and protein occurs. The highest pH recorded (Chapter 5) in the digesta of lambs was 7.89. To achieve a pH 10 in the SDS mix of the Perez-Maldonado (1994), Tri-ethanolamine was used as the buffer. In comparison, Tris–HCl was the choice of buffer in the modified method of Terrill et al. (1992b), this being criticised by Perez-Maldonado (1994) due to the possibility that HCl may interfere with the assay and give an underestimation of the concentration. When preparing solutions of Tris-HCl, addition of HCl was not required as the pH was correct without requiring adjustment and also the assay solution for analysis of CT fractions contains 5% HCl, so there is always HCl present in the assays including that of Perez-Maldonado (1994). If these solutions prove to be comparable then the safer solution of Perez-Maldonado (1994) should be used.

Data from Table 4.5 shows that the different SDS protein-bound CT extraction mixes resulted in significantly different ($P < 0.001$) protein-bound CT concentrations in the samples analysed. The modified method of Terrill et al. (1992b) resulted in the higher protein-bound CT concentration of 1.54 ± 0.10 % DM compared to 0.82 ± 0.03 % DM for the method of Perez-Maldonado (1994). Previous to the SDS protein-bound CT extraction step, the samples were treated identically for free CT extraction by the modified method of Terrill et al. (1992b), ruling out any other sources of difference. This result was consistently found in every analysis that was carried out to compare the two methods, and also found when comparing the rumen digesta samples in section 4.4.5 (Table 4.6). Therefore, the SDS extraction mix to use is that of the modified Terrill et al. (1992b), even though the concentration of 2-mercaptoethanol is higher than the concentration used in the method of Perez-Maldonado (1994).

4.5.3.3 Comparison of the number and length of SDS extractions used for the determination of protein-bound CT in calliandra using the methods of Perez-Maldonado (1994) and the modified method of Terrill et al. (1992b).
In the modified method of Terrill et al. (1992b) there are two 45 min incubations during the extraction of protein-bound CT, whereas, the method of Perez-Maldonado (1994) has a single 75 min incubation. If results are comparable the single extraction would be desirable due to considerations of time.

Results from this comparison showed that irrespective of the method of analysis used and therefore the different SDS extraction mixes, the two 45 min extractions were significantly higher ($P < 0.001$) in concentration of protein-bound CT (Table 4.5). This double extraction is more time consuming and uses twice the chemicals as the single extraction, but the difference in the CT concentrations is considerable and hence cannot be overlooked. The protein-bound CT concentrations are 1.4 and 1.6 times higher with the modified method of Terrill et al. (1992b) and Perez-Maldonado (1994), respectively, for samples extracted twice compared with once. Therefore the double extraction of protein-bound CT using the modified method of Terrill et al. (1992b) would be the desirable protocol to follow.

4.5.4 Analysis of fibre-bound CT

4.5.4.1 Comparison of the successive methanol then butanol washes versus no wash used for the determination of fibre-bound CT in calliandra using the methods of Perez-Maldonado (1994) and the modified method of Terrill et al. (1992b).
The method of Perez-Maldonado (1994) contains a successive methanol and butanol wash to eliminate SDS residues to convert the diluent for the fibre-bound CT standards from water to methanol. This wash step is absent in the modified method of Terrill et al. (1992b). If results are comparable then no wash would be faster, as it would eliminate time, chemicals and an extra set of standards from the analysis.

Results from this comparison showed fibre-bound CT concentration to be significantly higher in samples that were subjected to the methanol and butanol washes irrespective of SDS extraction mix or number and duration of extractions. The calculated total CT from these samples was only slightly higher than the samples

with no wash, but did not prove to be significant (Table 4.5). In hindsight adding this step into the modified method of Terrill et al. (1992b) would prove beneficial. In all previous analysis (Table 4.2) and from analysis of results not presented comparing the two methods the protein-bound CT and fibre-bound CT concentrations have been reciprocal. I was however assuming that this was due to the protein-bound CT not dissociating effectively with the Perez-Maldonado (1994) method and therefore was being carried over and detected in the fibre-bound CT sample as the total CT were never significantly different (Table 4.2 and 4.5, P = 0.973 and P = 0.222, respectively).

4.5.5 Comparison of the methods of Terrill et al. (1992b) and Perez-Maldonado (1994) using rumen digesta

Comparisons were also made between the two methods using rumen digesta samples collected from calliandra fed lambs. The modified method of Terrill et al. (1992b) was used to obtain the values for the free CT, after which the two separate methods were used for the comparison of the protein and fibre bound CT (Table 4.6). Results for the concentration of CT in rumen digesta (Table 4.6) agree with the results for protein and fibre-bound CT concentrations in calliandra and desmanthus (Table 4.2 and 4.5). The concentration of total CT calculated from the CT fractions was higher for the digesta analysed by the method of Perez-Maldonado (1994) as in all the analysis, however, for the digesta the values were significantly different, which was not the case in the analysis of samples from plant origin.

4.5.6 Determination of suitable blanks

Several different locally available plant samples were analysed for CT using the modified method of Terrill et al. (1992b) to determine the most suitable blank when analysing samples from plant material (Table 4.7). Rumen digesta from lambs that had previously been fed a diet of either lucerne pellets or fresh calliandra were analysed to determine if lucerne digesta could be used as a suitable blank when analysing digesta samples.

The analysis calls for a suitable blank to be used due to any background colour and impurities, therefore different readily available plants and the digesta were analysed for CT to determine the most suitable blanks. Lucerne contains trace amounts of CT at 1.4 % DM, which were found to be only slightly higher, but comparable with concentrations being reported as 0.16-1.2% DM (Douglas et al. 1995) and 0.05% DM (Jackson et al. 1996b). *Albizia lebbeck* had the lowest total CT concentration at 0.35% DM of all the plants analysed. Condensed tannin has been reported as containing 0.09% DM (free-CT not detected, protein-CT 0.06% and fibre-bound CT 0.03%) (Balogun et al. 1998). An appropriate blank decided upon was *Albizia lebbeck* for analysis of plant samples and rumen digesta from lucerne fed lambs..

4.6 Conclusion

From this study it can be concluded that analysis of plant material for total CT using either the method of Perez-Maldonado (1994) or modified method of Terrill et al. (1992b) would give comparable results, with values from analysis of calliandra and desmanthus not being significantly different. However, if the three fractions of CT, free, protein-bound and fibre-bound, are analysed using the two different methods as described in this study, then the modified method of Terrill et al. (1992b) would give a higher result for analysis of plant material. There were significant differences in the two methods for the fractions of CT when analysing lamb digesta. The modified method of Terrill et al. (1992b) gave a significantly higher result for protein-bound CT compared with the result for analysis with the method of Perez-Maldonado (1994). The fibre-bound-CT and calculated total CT, however, did result in a significantly higher value when analysed with the method of Perez-Maldonado (1994) compared with the modified method of Terrill et al. (1992b).

CHAPTER 5 THE INFLUENCE OF GASTROINTESTINAL NEMATODES AND HIGH TANNIN FEED ON pH AND CONDENSED TANNIN COMPLEXING IN THE GASTROINTESTINAL TRACT OF LAMBS.

5.1 Introduction

A significant problem in the ruminant industry is GI parasitism, with animal productivity losses and even death. A major concern to this industry is the increasing resistance by GI nematodes to the range of commercially available anthelmintics.

A novel strategy with potential is the inclusion in ruminant diets of plants that contain natural anthelmintics. This has the potential to offer a cost effective, possibly sustainable and an environmentally friendly option, which is likely to be attractive to farmers; particularly farmers in developing countries.

Initial work with particular temperate species *Lotus corniculatus*, *Lotus pedunculatus* (Robertson et al. 1995; Neizen et al. 1998a, Molan et al. 2002; Molan and Faraj, 2010), *Onobrychis viciifolia* (Paolini et al. 2004; Novobilsky et al. 2011) and *Hedysarum coronarium* (Neizen et al. 2002; Aissa et al. 2015) suggest that these plants have an anthelmintic effect. These temperate species have high concentrations of CT which is the compound presumed to be responsible for the anthelmintic effects. Investigations are ongoing into the anthelmintic effects of CT with many reports of the effects of sub-tropical and tropical species, *Lespedeza cuneata* (Burke et al. 2012), *Desmodium intortum* (Debela et al. 2012) and the shrub legume calliandra (Cresswell 2007).

Many studies have shown some or no effect of CT on larval development, migration, exsheathment, or egg production (Table 2.11). Recent research into tropical shrub legumes (Cresswell 2007) has found that feeding calliandra to lambs decreased egg

production in animals infected with *T. colubriformis* and *H. contortus* by 24-68% and 64-84%, respectively.

Cresswell (2007) concluded that:

"The results of the current experiment were in general agreement with the results of the pilot confirmed that calliandra, when fed to parasitised lambs over the course of an infection, reduced egg production by female *H. contortus* and *T. colubriformis*, but had no effect on worm burdens. The sex ratios of the worms were also unaffected. Because there were no improvements in the protein nutrition of the calliandra-fed lambs relative to the lucerne-fed lambs, improvements in resilience and resistance would not have occurred, indicating that calliandra had a direct physiological or toxic effect on the worms. The active component of calliandra could not be identified from this experiment, but it is probably condensed tannin."

Calliandra also has a high quality amino acid profile (Widiawati and Teleni 2004). A significant proportion of the protein from the legume is excreted as faeces possibly due to the high tannin content of calliandra (Cresswell, 2007). The potential for further improving the nutritional value of this legume together with its possible anthelmintic properties (high tannin) give calliandra added dimension as a feed.

The mechanisms by which the CTs are having anthelmintic effects are however unknown. Binding and dissociation of CTs with protein is pH dependant (Jones and Mangan 1977, Hagerman and Butler 1981). The pH of digesta in the different segments changes along GI tract, therefore it is likely that binding and dissociation of the different fractions will change along GIT due to pH changes. Experimental work will determine if pH of digesta and the complexing with the different fractions, free, protein-bound and fibre-bound CT is having any effect in the different segments of the GI tract in which nematodes inhabit. The outcomes may also help to explain why protein escapes degradation. It will also be determined if the presence of nematodes in the gut have any effect on pH and hence on binding and dissociation of the CT fractions.

This chapter will examine the dynamics of binding and dissociation of CT in the GI tract of lambs to determine if pH of digesta and complexing with the different fractions of CT are having any effects in the different segments which are inhabited by GI worms. The pH of the different segments of the digestive tract and the concentration of the CT fractions (protein-bound, fibre-bound and free-CT) in digesta of lambs fed calliandra or lucerne pellets (pH only) and infected with or without nematodes will be determined.

It was hypothesised that

i. *there is no difference in pH of GI tract segments due to the presence of either species of nematodes*
ii. *there is no difference in pH of GI tract segments due to a diet rich in CT or no CT (ie lambs fed either a diet of calliandra or lucerne)*
iii. *CT binding with and dissociation from protein and fibre along the GI tract is pH dependant*
iv. *CT binding with and dissociation from protein and fibre along the GI tract differs depending on GI segment.*

5.2 Materials and Methods

The animals used in this study were the same animals used in the study reported by Cresswell (2007). This experimental work involving animal care, management, and feeding and worm dosage was conducted by Cresswell (2007), my involvement was minimal until preparation for and during sampling days.

5.2.1 Experimental ethics

The experiment was conducted with Experimental Ethics Approval No A826_03 and in accordance with the *Guidelines for Housing and Care of Laboratory Animals* issued by The Experimental Ethics Review Committee of James Cook University.

5.2.2 Location

This experiment was undertaken in the Nutritional Physiology and Metabolism sheep shed and laboratory at the College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville.

5.2.3 Animals and management

5.2.3.1 Animals and housing

Tropical merino ram lambs were obtained from a sheep station at Richmond in Western Queensland when they were three months old and one day post weaning. Upon arrival at James Cook University they were weighed, ear-tagged and vaccinated with Ultravac 5 in 1 (CSL Ltd, Parkville Victoria) and drenched with both NILVERM LV oral drench (Coopers Animal Health, North Ryde, NSW, containing 80g/L Levamisole hydrochloride) at the rate of 1 mL/10kg live weight and Alben oral drench (Virbac Australia Pty Ltd, Peakhurst, NSW, containing 19g/L Albendazole).

The lambs were temporarily housed in cement floor pens and fed lucerne pellets (Lockyer Lucerne, Gatton, Queensland) until the adaptation period started when they were then moved to individual wooden slatted floor pens (2003, Period 1) or metal mesh floor pens (2004, Period 2). Pens were hosed out daily to remove faeces and urine. Animals had access to clean drinking water at all times and were checked daily for signs of ill-health.

5.2.3.2 Feeds and feeding

Animals were fed lucerne pellets initially *ad libitum* for a seven day period until DM intake was greater than 3.5% of live weight, after which intake was reduced to 2.8% of live weight so as all the feed was ensured to be consumed. Experimental diets were gradually introduced after 14 days. In both the lucerne and calliandra diets, rumen-undegradable protein in the form of casein was added, so as to match the amount of total protein in the calliandra diet. The grade-two acid casein was obtained from Malanda Milk in Mareeba, North Queensland. Half of the casein was treated with formaldehyde to produce rumen-undegradable protein (Hemsley et al. 1973). Formaldehyde (10% w/v) was added to the casein at a ratio of 100 mL formaldehyde to 1 kg of casein using an industrial mix master after which it was sealed in plastic bags for at least 12 days prior to feeding. The fresh calliandra leaves used in this experiment were harvested from plots close to the sheep shed at James Cook University. The average age of the legumes was 5-8 months regrowth. Feeds were offered twice daily at 08:30 and 17:00, with access to fresh water at all times.

Individual lambs had access to mineral blocks (Fosforlic mineralised stock block, Ridley Agriproducts Pty. Ltd., Queensland, Australia). Mineral composition is presented in Table 5.1.

Table 5.1 Composition of mineralised stock block offered to the experimental animals.

Mineral composition	%
Salt	75
Calcium	4.9
Phosphorus	1
Sulphur	2
Copper	0.06
Cobalt	0.006
Iodine	0.006
Zinc	0.1
Ferrous	0.11
Selenium	0.0005

5.2.3.3 Procedures

All animals were fully accustomed to the experimental procedures before sampling and measurement periods.

5.2.4 Experimental design

Forty lambs, approximately five months old were used in a Randomised Block Design. The experiment was divided into two periods, with a five month break to allow sufficient calliandra regrowth as the amount of fresh calliandra production was limited. Calliandra % DM, % CP and % OM were not significantly different ($P<0.05$) between periods (Cresswell, 2007). Lambs were randomly divided into four groups in 2003 and six groups in 2004, matched for live weight, as shown in Table 5.2.

Table 5.2 Lambs on either a diet of lucerne pellets or calliandra infected with either of the worm species *T. colubriformis* (Tc) or *H. contortus* (Hc) or no worm infection (controls) over two experimental periods.

	Period 1				Period 2					
Diet	lucerne		calliandra		lucerne			calliandra		
Worm Sp.	Tc	Hc	Tc	Hc	Tc	Hc	nil	Tc	Hc	nil
No. lambs	4	4	4	4	4	4	4	4	4	4

5.2.5 Worm Dose

After a one to two-week dietary adaptation period, lambs were orally dosed with the nematode species and fed for a further four weeks (lambs infected with *T. colubriformis*, produce eggs after three weeks) or five weeks (lambs infected with *H. contortus* produce eggs after three-four weeks). Lambs in the *T. colubriformis* group received 10 000 larvae and those in the *H. contortus* group received 3,200 larvae orally. The larvae were purchased from the CSIRO McMasters Laboratory, NSW. Four weeks after infection the lambs with *T. colubriformis* were humanely slaughtered. Lambs with *H. contortus* were slaughtered five weeks after infection.

5.2.6 Samples and measurements

Immediately after the lambs were slaughtered humanely with a captive bolt, the digestive tract was tied off and removed quickly. The GI tract was transferred into a large plastic tray and taken into an air-conditioned room where the segments were identified and tied off. The segments sampled were: rumen, abomasum, duodenum (0-4m from the pyloric sphincter), jejunum (4-5m from the pyloric sphincter), ileum (5m from the pyloric sphincter to the ileocecal valve), caecum and colon. Caecum and colon samples were not collected during Period 1. pH was measured by inserting a IJ44 Intermediate junction electrode (Ionode, Tennyson, QLD) into a small incision made in the GI tract segment and the reading recorded from the TPS digital LC80A pH meter (TPS Pty Ltd, Brisbane, QLD). The pH measurements were taken at mid-rumen abomasum and caecum, proximal duodenum, jejunum and ileum and in the spiral colon. After pH measurements were completed the incision was sutured and the contents of the individual segments emptied into beakers or measuring cylinders

and volume recorded. Digesta was mixed thoroughly, and a 15 mL sub-sample immediately placed on ice and subsequently stored at -20 ºC until analysis. Rarely was a large enough sample of jejunum able to be collected for analysis of CT. Total volume of digesta in each segment was recorded for use in a separate study (Cresswell 2007).

5.2.6.1 Dry matter

The frozen vials of digesta were placed into a container with silica gel and weighed quickly to establish a wet weight. The vials were place in a -80 ºC freezer for a minimum of 3 h after which the samples were freeze dried (Dynavac, Dynavac Engineering Pty, Ltd, Sydney) for approximately 31 h and weighed again to establish the dry weight.

5.2.6.2 Condensed tannin

The freeze dried samples were ground in a kitchen stainless steel Mini Moulin food mill with a 1mm mesh size. The ground samples were stored desiccated at -20 ºC until analysis. Samples were analysed using the modified method of Terrill et al. (1992b) as described in Chapter 4. Only 4 out of the eight lambs sampled (two from each period) for each worm species were analysed for CT due to time constraints to analyse the large number of samples. All four calliandra controls were analysed.

5.3 Statistical Analysis

Data were tabulated using Microsoft Office Excel 2003 (Microsoft Corporation, USA) and analysed using SPSS Version 13.0 for Windows (SPSS Inc., USA). Graphs were generated in Microsoft Office Excel 2003 (Microsoft Corporation, USA).

One-way analysis of variance was use to initially identify and rule out any factors that were not significant and were excluded from subsequent analysis. There was an age difference in the lambs of up to 2 months, which was beyond our control and the experiment was conducted over 2 periods. Initial analysis for these 2 factors as well as diet and worms were performed.

For analysis of pH, data were subjected to a MANOVA comparing diet and worm effects across all segments of the GI tract. Where the MANOVA showed significant effects of treatments, the mean values were compared using the least significant difference (LSD) test (Steel and Torrie, 1980).

Comparison of worm species (*T. colubriformis, H. contortus* and uninfected controls) for the three fractions of CT in digesta from the GI tract, Kruskall-Wallace tests was used to identify effects and then significance tested using Mann-Whitney test. Analysis for comparison of overall means of the three fractions of CT and total CT across segments of the GI tract as well as feed from lambs pooled for worms and uninfected and fed the calliandra diet was performed using Kruswell-Wallace and then Mann-Whitney test to determine significance.

A Mixed Model using reml in SPSS was used to explore the relationship between pH and fractions of CT and relationships between fractions of CT. The regression was adjusted for worm, segment and animal effects and coefficient reported with 95% confidence intervals.

5.4 Results

5.4.1 Dry matter

The results of DM analysis of the digesta collected and analysed from each segment of the GI tract and faeces are presented in Table 5.3.

Table 5.3 Means ± standard error of the means of dry matter (DM) of digesta from rumen, abomasum, duodenum, jejunum, ileum, caecum, colon and faeces across all experimental lambs.

Digesta	**n**	**% DM**
Rumen	39	44.71 ± 0.46
Abomasum	40	45.38 ± 0.41
Duodenum	31	46.89 ± 0.92
Jejunum	8	47.33 ± 1.66
Ileum	40	47.16 ± 0.41
Caecum	25	47.40 ± 0.35
Colon	25	54.60 ± 0.63
faeces	40	81.42 ± 0.50

5.4.2 Worm numbers present in abomasum and small intestine

Total worm number of *H. contortus* and *T. colubriformis* present in the abomasum and small intestine of the lambs respectively are presented in Table 5.4. Data are from Cresswell (2007).

Table 5.4 Total number of worms, estimated egg output in *H. contortus* and *T. colubriformis* harvested immediately after euthanasia of lambs that were infected with either of the two worm species and fed either lucerne pellets or calliandra. Values are means ± standard errors. Worm numbers were not significantly different for each species.

	H. contortus		*T. colubriformis*		Uninfected controls*	
	lucerne	calliandra	lucerne	calliandra	lucerne	calliandra
Total worms	1861 ± 230	2237 ± 395	5718 ± 339	4862 ± 452	0	0
Eggs (no./female worm/24h)	3299 ± 560	274 ± 65	134 ± 15	57 ± 21	0	0

From: Cresswell (2007); * Cresswell 2007, unpublished data

5.4.3 pH of digesta

Data for pH values of each segment of the GI tract for lambs on either a diet of lucerne or calliandra and infected with either of the worm species, *T. colubriformis* or *H. contortus* or no worm infection are presented in Table 5.5.

Table 5.5 Means ± standard error of the means for pH of digesta from gastrointestinal tract segments of lambs on either a diet of lucerne pellets or calliandra and infected with either *T. colubriformis* (Tc) or *H. contortus* (Hc) or no worm infection (controls).

Diet	lucerne			calliandra			Diet effect	Worm species effect	Diet x Worm effect
Worm sp.	Tc	Hc	controls	Tc	Hc	controls	P	P	P
Digesta:									
Rumen	6.82 ± 0.06	6.84 ± 0.04	6.77 ± 0.04	6.91 ± 0.09	6.81 ± 0.03	6.77 ± 0.07	0.675	0.232	0.518
Abomasum	2.92 ± 0.12	2.95 ± 0.20	3.40 ± 0.18	4.04a,b ± 0.50	4.58a ± 0.35	3.41b ± 0.25	0.000	0.374	0.018
Duodenum	5.62 ± 0.31	6.25 ± 0.12	5.25 ± 0.39	5.53 ± 0.26	5.10 ± 0.53	4.69 ± 0.50	0.075	0.172	0.414
Jejunum	6.15 ± 0.16	6.30 ± 0.12	6.34 ± 0.06	6.61 ± 0.19	6.39 ± 0.18	6.27 ± 0.10	0.188	0.887	0.190
Ileum	7.43a ± 0.08	7.07b ± 0.15	7.37 ± 0.04	7.52 ± 0.08	7.42 ± 0.11	7.59 ± 0.03	0.009	0.031	0.361
Caecum	6.71a ± 0.04	6.79 ± 0.03	6.94b ± 0.06	7.06 ± 0.06	7.10 ± 0.06	7.15 ± 0.07	0.000	0.030	0.437
Colon	6.93a ± 0.03	7.11b ± 0.04	7.13b ± 0.04	7.15 ± 0.08	7.19 ± 0.05	7.35 ± 0.06	0.000	0.003	0.300

Values within a row for each diet with different letters differ significantly ($P < 0.05$).

Data from the analysis of digesta sampled from the GI tract of lambs shows that both worm and diet had an effect on the digesta pH (table 5.5). The pH of digesta was higher in the lambs that were infected with worms compared to the uninfected controls. There was a significant effect due to the interaction of worm and diet in the abomasum ($P = 0.018$) due to higher pH values for the calliandra diet with worm infections.

Data for pH values of each segment of the GI tract for lambs on either a diet of lucerne or calliandra and pooled for infected and uninfected with worms are presented in Table 5.6 and Figure 5.1.

Table 5.6 Means ± standard error of the means for pH of digesta from rumen, abomasum, duodenum, jejunum, ileum, caecum and colon of lambs fed lucerne pellets or calliandra. Data are pooled for worm infected and uninfected lambs.

Diet Digesta:	lucerne	calliandra	P value
Rumen	6.81a ± 0.03	6.82a ± 0.04	0.675
Abomasum	3.08a ± 0.11	4.01b ± 0.24	0.000
Duodenum	5.70a ± 0.20	5.07a ± 0.27	0.075
Jejunum	6.25a ± 0.07	6.40a ± 0.09	0.188
Ileum	7.30a ± 0.07	7.51b ± 0.05	0.009
Caecum	6.80a ± 0.04	7.11b ± 0.04	0.000
Colon	7.05a ± 0.03	7.24b ± 0.04	0.000

Values within a row with different letters differ significantly ($P < 0.05$).

Diet had a significant effect on pH values in the abomasum ($P < 0.001$), ileum ($P = 0.009$), caecum ($P < 0.001$) and colon ($P < 0.001$). These pH values were consistently higher in all these segments for lambs fed the calliandra diet.

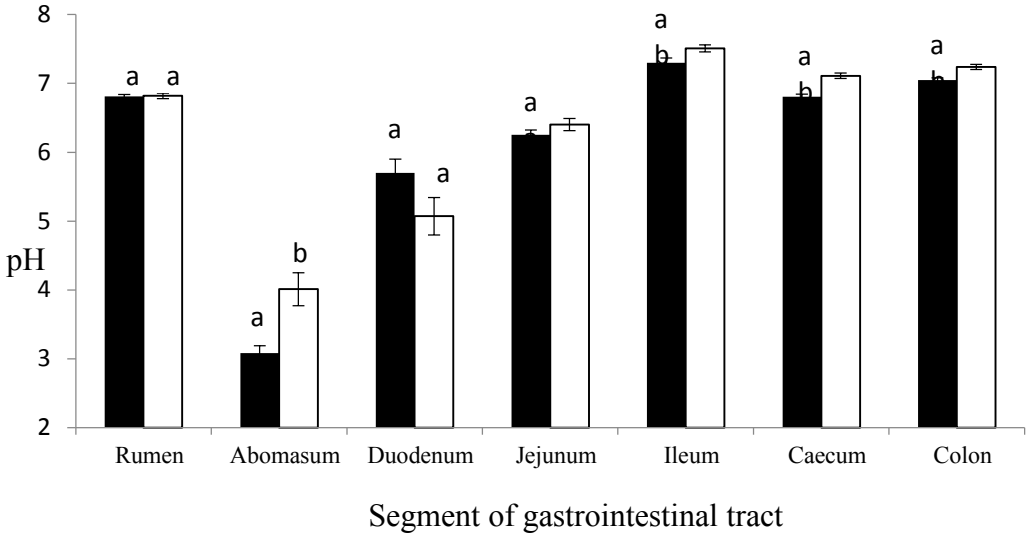


Figure 5.1 Means ± standard error of the means for pH of digesta from rumen, abomasum, duodenum, jejunum, ileum, caecum and colon of lambs fed a diet of either lucerne pellets (■) or calliandra (□and infected with either *T. colubriformis*, *H. contortus* or no worm infection (controls). Data are pooled for infected and uninfected. Within each GI segment, columns with different letters are significantly different ($P < 0.05$).

Data for pH values of each segment of the GI tract for lambs infected with either of the worm species, *T. colubriformis* or *H. contortus* or no worm infection and pooled for diet are presented in Table 5.7 and Figure 5.2.

Table 5.7 Means ± standard error of the means for pH of digesta from rumen, abomasum, duodenum, jejunum, ileum, caecum and colon of lambs infected with either *T. colubriformis*, *H. contortus* or no worm infection (controls).Data are pooled for diet.

Worm sp. Digesta:	*T. colubriformis*	*H. contortus*	Controls	P value
Rumen	6.85a ± 0.05	6.83a ± 0.02	6.77a ± 0.04	0.232
Abomasum	3.34a ± 0.27	3.76a ± 0.36	3.41a ± 0.14	0.374
Duodenum	5.59a ± 0.20	5.67a ± 0.33	4.97a ± 0.31	0.172
Jejunum	6.32a ± 0.14	6.34a ± 0.10	6.31a ± 0.05	0.887
Ileum	7.47a ± 0.06	7.25b ± 0.11	7.48a ± 0.05	0.031
Caecum	6.84a ± 0.07	6.94a,b ± 0.07	7.05b ± 0.06	0.030
Colon	7.01a ± 0.05	7.15b ± 0.03	7.24b ± 0.05	0.003

There were significant differences in pH values between the worm species and controls (no infection) in the ileum (P = 0.031), caecum (P = 0.030) and colon (P = 0.003), with these values being consistently lower with worm infection compared to the controls with no worm infection in these three segments. There were no differences in pH values between the worm species and controls (no infection) in the rumen (P = 0.232), abomasum (P = 0.374), duodenum (P = 0.172) or jejunum (P = 0.887). In the ileum there were significant differences in the pH values between *T. colubriformis* and *H. contortus* (P = 0.021) and the *H. contortus* and controls (P = 0.001), again the lower pH was with the worm infection compared to no infection. In the caecum, the significant differences were between the *T. colubriformis* and controls (P = 0.001), again the lower pH was with the worm infection compared to no infection. The differences in pH in the caecum are approaching significance between the *T. colubriformis* and *H.contortus* and the *H. contortus* and controls. In the colon differences in the pH values were between *T. colubriformis* and *H. contortus* (P = 0.010) and between *T. colubriformis* and controls (P < 0.001), with the lowest pH values recorded for the infection with *T. colubriformis.*

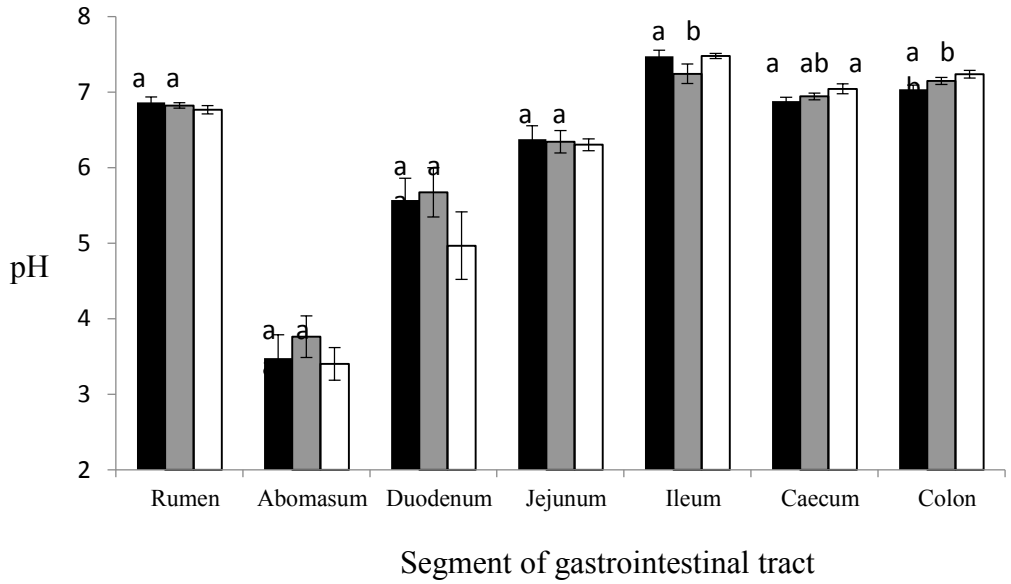


Figure 5.2 Means ± standard error of the means for pH of digesta from rumen, abomasum, duodenum, jejunum, ileum, caecum and colon of lambs infected with either *T. colubriformis* (■), *H. contortus* (▒) or no worm infection (□). Data are pooled for diet. Within each GI segment, columns with different letters are significantly different ($P < 0.05$).

Data for pH values of each segment of the GI tract for lambs the worm species, pooled for diet and worm species are presented in Table 5.8 and Figure 5.3.

Table 5.8 Overall means ± standard error of the means for pH of digesta from rumen, abomasum, duodenum, jejunum, ileum, caecum and colon of lambs. Data are pooled for diet and worm species.

Digesta:	pH
Rumen	6.82 ± 0.02
Abomasum	3.50 ± 0.16
Duodenum	5.41 ± 0.17
Jejunum	6.32 ± 0.06
Ileum	7.40 ± 0.05
Caecum	6.94 ± 0.20
Colon	7.13 ± 0.03
p value	0.000

There were significant differences in pH values between the segments of the GI tract ($P < 0.001$).

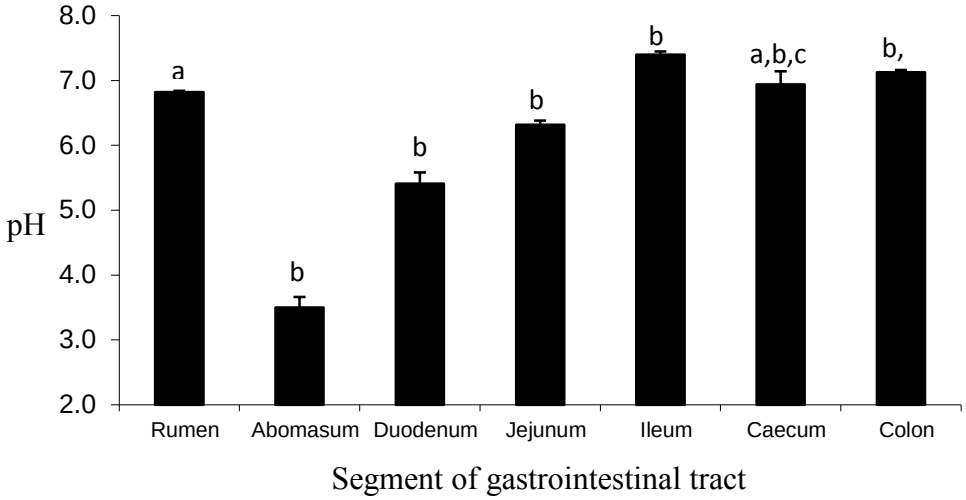


Figure 5.3 Overall means ± standard error of the means for pH of digesta from rumen, abomasum, duodenum, jejunum, ileum, caecum and colon of lambs. Data are pooled for diet and worm species. Columns with different letters are significantly different ($P < 0.05$).

The pH values of each segment of the GI tract for lambs differed with worm species ($P < 0.001$). Rumen pH was similar to that in the caecum ($P = 0.288$) but different to the pH in the abomasum ($P < 0.001$), duodenum ($P < 0.001$), jejunum ($P < 0.001$), ileum ($P < 0.001$) and colon ($P = 0.011$). Abomasal, duodenal, ileal pH was significantly different to pH in all GI segments ($P < 0.001$). Jejunum pH was different to caecum ($P < 0.001$) and colon ($P = 0.012$). In addition caecum and colon pH values were similar ($P = 0.183$).

5.4.4 Condensed tannin concentration in digesta

The concentration of the free, protein-bound, fibre-bound and total CT for the two feeds, lucerne pellets and calliandra used in this experiment are presented in Table 5.9 (from chapter 4).

Table 5.9 Concentration of free, protein-bound, fibre-bound and total condensed tannin as % dry matter (DM) in calliandra and lucerne pellets.

	Condensed Tannin (% DM)			
Plant	Free	Protein-bound	Fibre-bound	Total
calliandra	9.48	1.21	0.95	12.00
lucerne pellets	0.48	0.74	0.18	1.40

The concentration of CT in each GI segment of the lambs is presented in Table 5.10. Volume data were obtained from (Cresswell 2007).

Table 5.10 Condensed tannin (CT) concentration [g/L digesta and % dry matter (DM)] ± standard error of the means in GI segments of lambs fed a diet of calliandra and uninfected with worms.

GI segment	Rumen	Abomasum	Duodenum	Ileum	Caecum	Colon
CT (g/L)	19.1 ± 0.9	52.7 ± 2.0	9.0 ± 1.3	26.0 ± 0.8	29.6 ± 0.8	31.4 ± 1.2
CT (% DM)	4.06 ± 0.19	10.77 ± 0.41	7.86 ± 0.26	5.21 ± 0.16	6.02 ± 0.16	5.97 ± 0.08

The free, protein-bound, fibre-bound and total CT (calculated) means ± standard error of the means for each segment of the GI tract as well as the feed are presented in Table 5.11. This data is from calliandra fed lambs and does not include any values for jejunum as an adequate sample size was not collected.

Table 5.11 Means ± standard error of the means for concentration of free, protein-bound, fibre-bound and total condensed tannin in digesta from rumen, abomasum, duodenum, ileum, caecum and colon of lambs fed a diet of calliandra and infected with either *T. colubriformis, H. contortus* or no worm infection (controls).

Condensed tannin concentration (% DM)				
Worm species	*T. colubriformis*	*H. contortus*	nil	Overall Means
Feed				
Free CT	9.91	9.91	9.91	9.91
Protein-bound CT	1.20	1.20	1.20	1.20
Fibre-bound CT	0.84	0.84	0.84	0.84
Total CT	11.95	11.95	11.95	11.95
Rumen				
Free CT	1.5 ± 0.27	1.85 ± 0.30	0.78 ± 0.11	1.38 ± 0.14
Protein-bound CT	2.40 ± 0.41	2.62 ± 0.32	2.33 ± 0.11	2.45 ± 0.15
Fibre-bound CT	0.87 ± 0.08	1.05 ± 0.06	0.95 ± 0.05	0.96 ± 0.04
Total CT	4.77 ± 0.74	5.52 ± 0.59	4.06 ± 0.19	4.79 ± 0.28
Abomasum				
Free CT	11.84 ± 0.73	11.22 ± 0.91	10.04 ± 0.46	11.04 ± 0.36
Protein-bound CT	0.06 ± 0.04	0.22 ± 0.08	0.35 ± 0.07	0.22 ± 0.04
Fibre-bound CT	0.17 ± 0.03	0.45 ± 0.09	0.38 ± 0.03	0.33 ± 0.04
Total CT	12.08 ± 0.75	11.91 ± 0.84	10.77 ± 0.41	11.59 ± 0.34
Duodenum				
Free CT	1.36 ± 0.31	3.04 ± 0.62	0.74 ± 0.26	1.71 ± 0.26
Protein-bound CT	0.33 ± 0.18	0.29 ± 0.16	0.00 ± 0.00	0.20 ± 0.07
Fibre-bound CT	1.28 ± 0.16	1.12 ± 0.08	1.12 ± 0.05	1.17 ± 0.05
Total CT	2.97 ± 0.43	4.45 ± 0.78	1.86 ± 0.26	3.09 ± 0.31
Ileum				
Free CT	1.18 ± 0.24	1.55 ± 0.20	0.45 ± 0.08	1.06 ± 0.12
Protein-bound CT	1.60 ± 0.30	1.59 ± 0.15	2.17 ± 0.12	1.76 ± 0.10
Fibre-bound CT	2.82 ± 0.12	3.06 ± 0.26	2.59 ± 0.07	2.81 ± 0.07
Total CT	5.60 ± 0.45	6.24 ± 0.52	5.21 ± 0.16	5.63 ± 0.20
Caecum				
Free CT	1.07 ± 0.12	0.59 ± 0.20	0.00 ± 0.00	0.48 ± 0.11
Protein-bound CT	2.49 ± 0.24	2.21 ± 0.17	2.78 ± 0.14	2.53 ± 0.08
Fibre-bound CT	2.71 ± 0.24	2.80 ± 0.16	3.25 ± 0.14	2.96 ± 0.10
Total CT	6.27 ± 0.18	5.23 ± 0.64	6.02 ± 0.16	5.87 ± 0.15
Colon				
Free CT	0.83 ± 0.14	0.43 ± 0.19	0.11 ± 0.03	0.38 ± 0.08
Protein-bound CT	1.94 ± 0.04	2.04 ± 0.04	2.87 ± 0.11	2.43 ± 0.09
Fibre-bound CT	2.91 ± 0.18	3.35 ± 0.19	3.14 ± 0.12	3.13 ± 0.07
Total CT	5.68 ± 0.12	5.81 ± 0.20	5.97 ± 0.22	5.86 ± 0.08

There were no significant effects ($P > 0.05$) due to the presence of either of the two worm species or with no worms on CT concentrations for the any of the fractions of CT in any of the GI segments.

5.4.5 Effect of GI segment on concentration of CT

There were no significant effects ($P > 0.05$) due to the presence of either worm species or no worms on concentrations for the any of the fractions of CT in any of the GI segments. Data pooled for worms and no worms to see overall effect between GI segments for each fraction of CT are presented in Table 5.12.

There was a significant effect of GI segment on the concentration of all fractions of CT for overall means, with $P < 0.001$ for all CT fractions and the calculated total CT.

Table 5.12 Means ± standard error of the means of data pooled for worms for concentration of free, protein-bound, fibre-bound and total condensed tannin (CT) as % dry matter (DM) in feed and digesta from rumen, abomasum, duodenum, ileum, caecum and colon of lambs fed a diet of calliandra.

	Condensed tannin concentration (% DM)			
GI Segment /Feed	**Free CT**	**Protein-bound CT**	**Fibre-bound CT**	**Total CT**
Feed	9.91b	1.20b,c	0.84a	11.95b
Rumen	1.38a,d ± 0.14	2.45a,d ± 0.15	0.96a ± 0.04	4.79a ± 0.28
Abomasum	11.04b ± 0.36	0.22b ± 0.04	0.33a ± 0.04	11.59b ± 0.34
Duodenum	1.71a,d ± 0.26	0.20b ± 0.07	1.17a ± 0.05	3.09a ± 0.31
Ileum	1.06a,d ± 0.12	1.76a ± 0.10	2.81a,b ± 0.07	5.63a,d ± 0.20
Caecum	0.48b,c,d ± 0.11	2.53a,d ± 0.08	2.96a,b ± 0.10	5.87d ± 0.15
Colon	0.38b,c,d ± 0.08	2.43a,d ± 0.09	3.13a,b ± 0.07	5.86d ± 0.08
P value	0.000	0.000	0.000	0.000

Columns with different letters are significantly different ($P < 0.05$).

For the free fraction of CT there were significant differences in the CT concentration between the feed and all GI segments ($P < 0.001$) except the abomasum ($P = 1.000$). The concentration of free CT in the rumen was significantly different to that in the abomasum ($P < 0.001$), caecum ($P = 0.036$) and colon ($P = 0.007$), but not different to that in the duodenum ($P = 0.932$) and ileum ($P = 0.443$). The concentration of free CT in the abomasum was significantly different to that in all the GI segments ($P < 0.001$). The concentration of free CT in the duodenum was not significantly different to that in the segments of the ileum ($P = 0.590$), caecum ($P = 1.000$), and colon ($P = 0.098$). The concentration of free CT in the ileum was not significantly different to

that in the caecum (P = 0.142), and colon (P = 0.057), and the concentration of free CT was not significantly different between the caecum and colon (P = 0.955).

For the protein-bound fraction of the CT there were significant differences in the CT concentration between the feed and all GI segments (P < 0.001; ileum P = 0.044). The concentration of protein-bound CT in the rumen was significantly different to that in the abomasum (P < 0.001) and duodenum (P < 0.001). The concentration of protein-bound CT in the rumen was not significantly different to that in the ileum (P = 0.114), caecum (P = 0.536) and colon (P = 0.970).The concentration of protein-bound CT in the abomasum was not significantly different to that in the duodenum (P = 0.319), but was different to all other GI segments (P < 0.001). The concentration of protein-bound CT in the duodenum was significantly different to that in all other GI segments (P < 0.001), except the abomasum (P = 0.319). The concentration of protein-bound CT in the ileum was significantly different to that in the caecum (P = 0.022) and colon (P = 0.039). The concentration of protein-bound CT was not significantly different between the caecum and colon (P = 0.613).

For the fibre-bound fraction of the CT there were significant differences in the CT concentration between the feed and all GI segments (P < 0.001), except the abomasum (P = 1.000). The concentration of fibre-bound CT in the rumen was significantly different to that in all the other GI segments (P < 0.001) except the duodenum (P = 0.089). The concentration of fibre-bound CT in the abomasum was significantly different to that in all the other GI segments (P < 0.001). The concentration of fibre-bound CT in the duodenum was significantly different to that in all other GI segments P < 0.001), except the rumen (P = 0.089). The concentration of fibre-bound CT in the ileum was not significantly different between that of the caecum (P = 0.432) or colon (P = 0.115). The concentration of fibre-bound CT in caecum was not significantly to that in the colon (P = 0.336).

For the calculated total CT there were significant differences in the CT concentration between the feed and all GI segments (P < 0.001), except the abomasum (P = 0.190). The concentration of total CT in the rumen was significantly different to that in the

abomasum (P < 0.001), caecum (P = 0.045) and colon (P = 0.016). The concentration of total CT in the rumen was not significantly different to that in the duodenum (P = 0.060) and ileum (P = 0.089). The concentration of total CT in the abomasum was significantly different to that in all the other GI segments (P < 0.001). The concentration of total CT in the duodenum was significantly different to that in the GI segments ileum (P = 0.001), caecum (P = 0.004) and colon (P = 0.001). The concentration of total CT in the ileum was not significantly different to that in the caecum (P = 0.432) and colon (P = 0.384). The concentration of total CT was not significantly different between that of the caecum and colon (P = 0.694).

5.4.6 Relationship between condensed tannin and pH

The relationships between pH and free CT and pH and total CT can be seen in figures 5.4 and 5.5, respectively. Regression has been adjusted for worm, segment and animal effects.

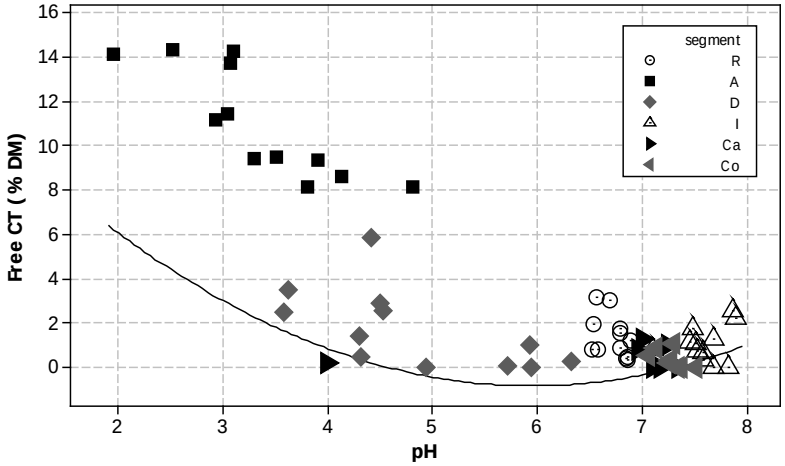


Figure 5.4 Relationship (R^2 = 0.196) between pH and free condensed tannin (CT) as % dry matter (DM) in the gastrointestinal tract segments, rumen (R), abomasum (A), duodenum (D), Ileum (I), caecum (Ca), and colon (Co).

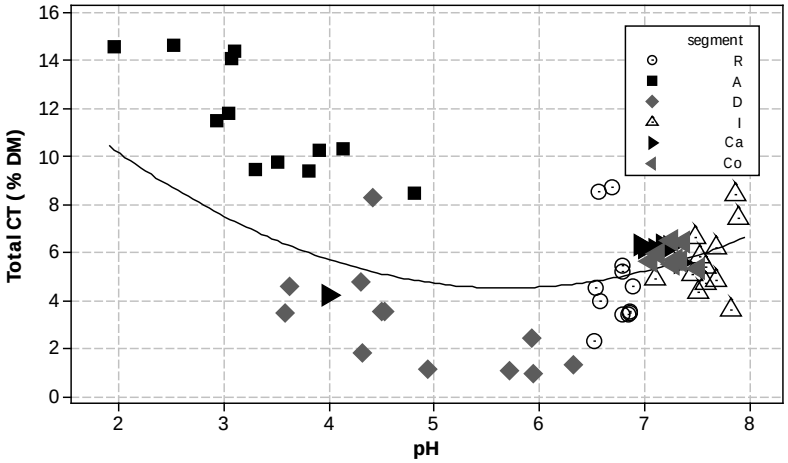


Figure 5.5 Relationship ($R^2 = 0.171$) between pH and total condensed tannin (CT) as % dry matter (DM) in the gastrointestinal tract segments, rumen, abomasum, duodenum, Ileum, caecum, and colon.

A significant quadratic relationship was found to exist between pH and free CT ($P < 0.001$; coefficient = 0.443) as well as between pH and total CT ($P = 0.005$; coefficient = 0.413). No significant relationships were found to exist between pH and protein-bound CT ($P = 0.510$) or between pH and fibre-bound CT ($P = 0.705$).

5.4.7 Relationships between CT fractions

The regression coefficients for the relationships between the fractions of CT are shown in Table 5.13 and Figures 5.6-5.10. Regression has been adjusted for worm, segment, CT fractions and animal effects.

Table 5.13 Regression coefficients for relationships between free, protein-bound, fibre-bound and total condensed tannin.

Condensed tannin fractions		Coefficient	P value	Linear/ Quadratic	Animal Variability	
					Residual	ID
Free	Protein-bound	0.467	0.102	L		
Free	Total	0.027	0.000	Q	1.3	0.8
Free	Fibre-bound	-0.667	0.144	L		
Protein-bound	Free	-0.014	0.019	Q	0.3	0.1
Protein-bound	Total	-0.018	0.001	Q	0.2	0.1
Protein-bound	Fibre-bound	0.318	0.146	L		
Fibre-bound	Free	-0.020	0.580	L		
Fibre-bound	Protein-bound	0.094	0.233	L		
Fibre-bound	Total	-0.007	0.083	Q		
Total	Protein-bound	1.576	0.000	L	0.6	0.0
Total	Free	-0.022	0.005	Q	1.2	1.0
Total	Fibre-bound	0.730	0.191	L		

There were significant relationships between free and total CT, protein-bound CT and both free and total CT, as well as between total CT and both protein-bound and free CT.

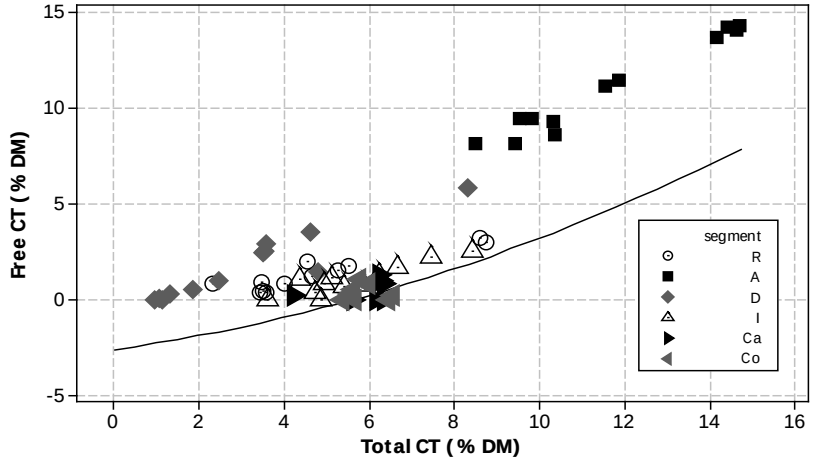


Figure 5.6. Relationship between free and total condensed tannin (CT) as % dry matter (DM) in the gastrointestinal tract segments, rumen (R) , abomasum (A), duodenum (D), Ileum (I), caecum (Ca), and colon (Co).

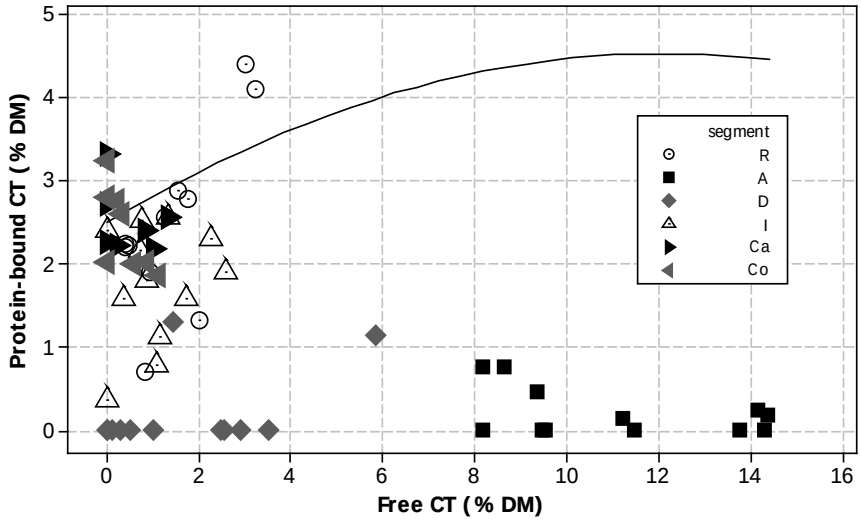


Figure 5.7 Relationship between protein-bound and free condensed tannin (CT) as % dry matter (DM) in the gastrointestinal tract segments, rumen (R), abomasum (A), duodenum (D), Ileum (I), caecum (Ca), and colon (Co).

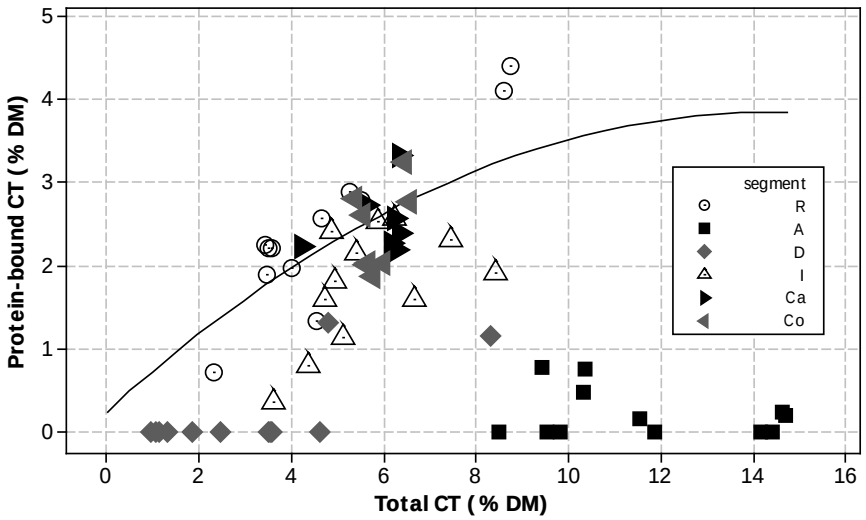


Figure 5.8 Relationship between protein-bound and total condensed tannin (CT) as % dry matter (DM) in the gastrointestinal tract segments, rumen (R), abomasum (A), duodenum (D), Ileum (I), caecum (Ca), and colon (Co).

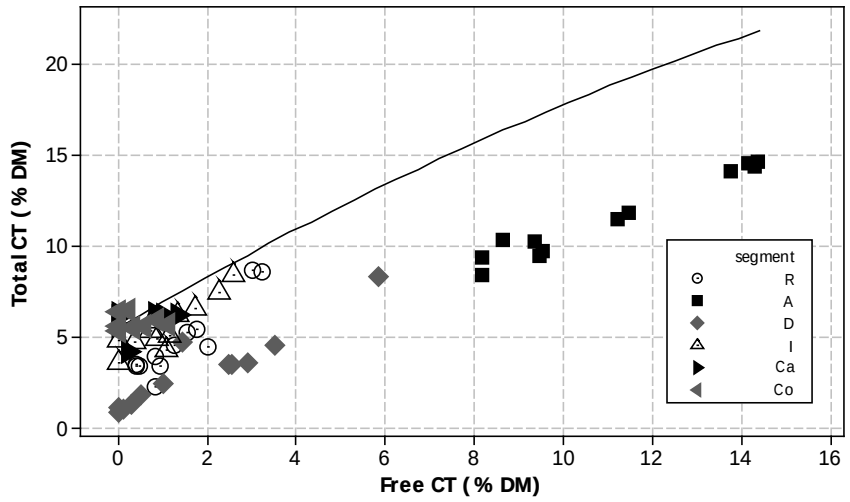


Figure 5.9 Relationship between total and free condensed tannin (CT) as % dry matter (DM) in the gastrointestinal tract segments, rumen (R), abomasum (A), duodenum (D), Ileum (I), caecum (Ca), and colon (Co).

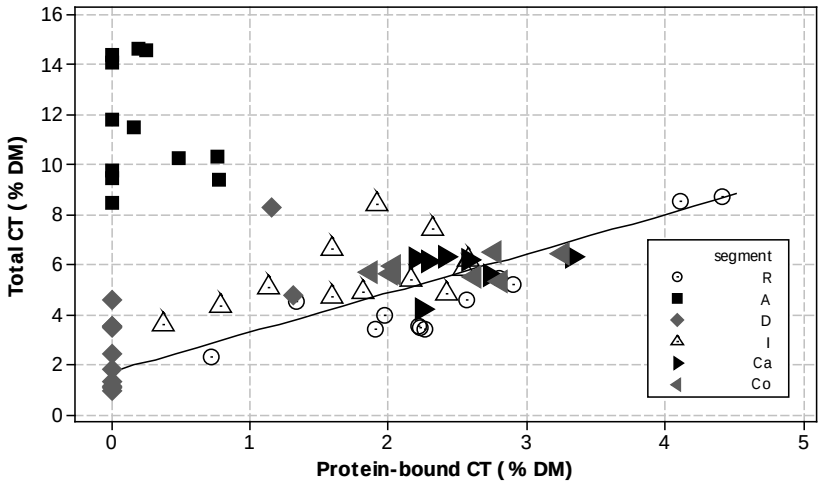


Figure 5.10 Relationship between total and protein-bound condensed tannin (CT) as % dry matter (DM) in the gastrointestinal tract segments, rumen (R), abomasum (A), duodenum (D), Ileum (I), caecum (Ca), and colon (Co).

5.4.8 Concentration and detection of CT along the gastrointestinal tract

Data for the percent of each fraction of CT that was detected in the CT assay compared to the CT present in the feed are presented in Table 5.14 and Figures 5.11 and 5.12.

Table 5.14 pH, total condensed tannin (CT) as % dry matter (DM) of feed and digesta from rumen, abomasum, duodenum, ileum, caecum, colon and faeces, % CT that was undetected or lost, % of each fraction as compared to total detected in each gastrointestinal segment.

	pH of digesta	Total CT	*CT (g/L)	% CT undetected or lost	% CT detected as compared to feed total			
					total	free	Protein-bound	Fibre-bound
Feed	-	12.00	-	0	100	82	10	8
Rumen	6.7	4.65	19	61	39	29	51	20
Abomasum	3.3	11.44	53	4	96	95	2	3
Duodenum	4.8	2.87	9	76	24	55	7	38
Ileum	7.6	5.58	26	53	47	19	31	50
Caecum	6.7	5.90	30	51	49	8	43	49
Colon	7.3	5.88	31	51	49	6	41	53
Faeces	-	5.44	-	54	46	3	47	50

*Volume data from (Cresswell 2007), for control worms only.

The total CT concentration in the feed and abomasum were comparable at 12.00 and 11.44 % DM, respectively. However, the concentration detected in all other GI segments was considerably lower than what was ingested. The lowest concentration of CT was in the duodenum and 74% less than what was ingested.

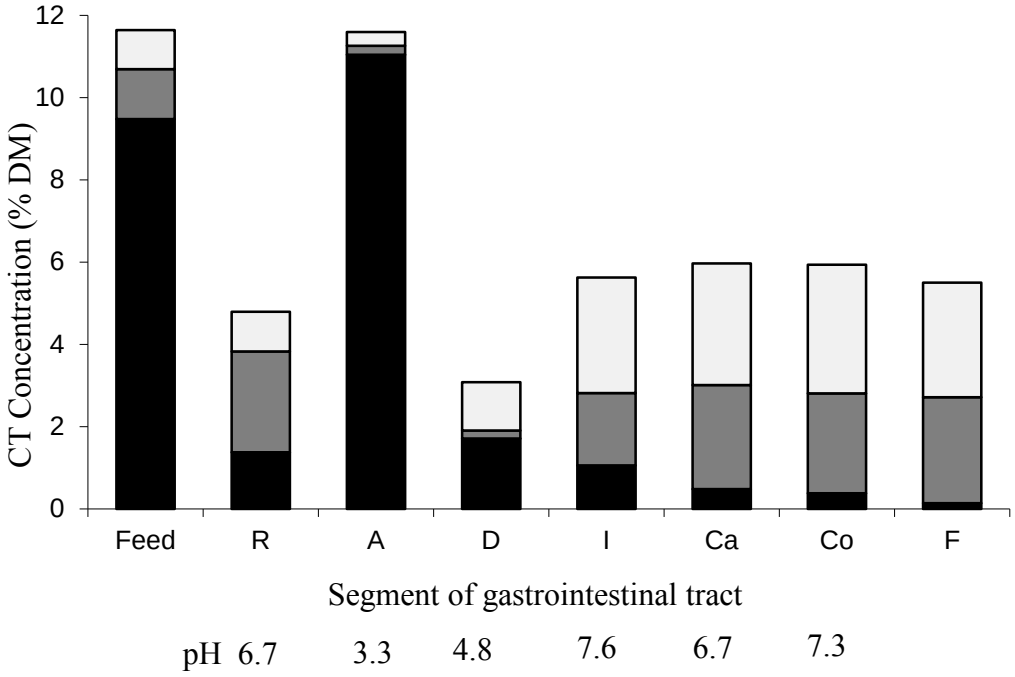


Figure 5.11 Means for total condensed tannin (CT) as % dry matter (DM) with the proportion of free (■), protein-bound (▩) CT and fibre-bound (□) CT fractions infeed and digesta from rumen (R), abomasum (A), duodenum (D), ileum (I), caecum (Ca) and colon (Co) of lambs fed calliandra. Data are pooled for worms and no worms. Faecal samples were collected at a different time to the digesta.

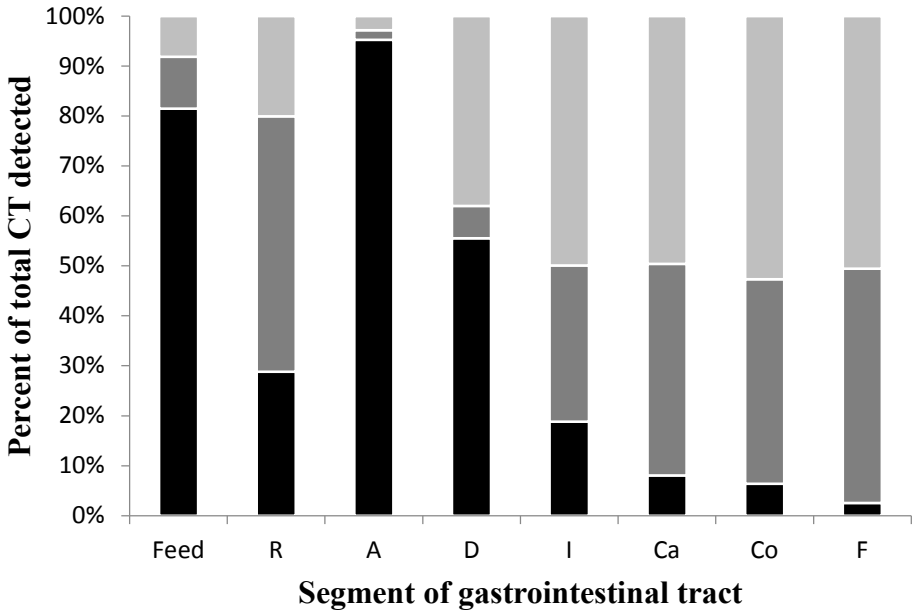


Figure 5.12 Free (■), protein-bound (▩) and fibre-bound (□) CT fractions as percentage of total CT detected in digesta from rumen (R), abomasum (A), duodenum (D), ileum (I), caecum (Ca) colon (Co) and faeces (F) of lambs fed calliandra compared with CT in feed. Data are pooled for worms and no worms. Faecal samples were collected at a different time to the digesta samples.

5.4.9 Condensed tannin in faeces

The concentrations of the three fractions of CT were also determined in faeces as seen in Figures 5.10 and 5.11. The faeces, however, were collected during the N balance experiment conducted by Cresswell (2007), a few days before slaughter, so cannot be compared with the GI tract samples as they were not collected at the same time. They are being reported here to show that the different fractions of CT are present and can be assayed in the faeces. The concentrations of the three fractions of CT are however, similar to that found in the lower GI tract.

5.5 Discussion

5.5.1 pH

pH was determined in GI segments to see if pH of digesta from lambs fed a diet that was either low or high in CT or infected with either of the worm species, *T. colubriformis* or *H. contortus* or uninfected might influence these values. It is well known that pH is one of the important factors in the formation of the CT complexes with protein as well as fibre in the GI tract. The amount of CT that is dissociated and available as free CT is also important if in fact CT binding to the worm larvae, adults or eggs has some contribution to its anthelmintic effects. Furthermore, the complexing of protein-bound CT influenced by pH is important to know if the protein is available in the rumen for microbe synthesis, or is protected in the rumen and then becomes available in the abomasum and duodenum for digestion and utilisation of amino acids by the animals.

Overall, there was a diet and worm interaction, which had a significant affect in the abomasum only, with the pH being higher in the digesta from calliandra fed lambs that were infected with both worms (Table 5.5). Although this is the segment that *H. contortus* inhabits, digesta from lambs infected with both worm species had a higher pH value. This effect is likely to be due to diet.

Diet had a significant effect on pH values in the abomasum, and the lower GI segments, ileum, caecum and colon (Table 5.6 and Figure 5.1). These pH values were consistently higher in all these segments for lambs fed the CT containing

calliandra compared with the lucerne diet. The largest pH difference can be seen in the abomasum with the mean pH for lucerne being 3.08 compared to the calliandra diet with pH of 4.01. It is not clear as to why the pH is higher in these segments for lambs fed the calliandra diet. The pH was almost identical in the rumen and tended to be slightly higher in the jejunum but lower in the duodenum, however, none of which were significantly different.

The pH of the digesta from the caecum from lambs that were infected with *T. colubriformis*, had significantly lower pH values compared to the controls. In the colon this was also the case, but the pH for the digesta from lambs infected with *H. contortus* were greater than that of the *T. colubriformis*. It has been shown that infection with *T. colubriformis* resulted in changes in digesta flow and N metabolism with smaller rumen volumes and larger abomasum volumes, small intestine and caecal-colon volumes, indicating a shift of fermentation caudally (Roseby 1977). Work by Rowe et al. (1988) also showed that infection with *H. contortus* caused a reduction in rumen digestion and altered fermentation with increased digesta flow and changes in the pattern of volatile fatty acid production, suggesting either altered motility or some effect on microbial populations.

Infections with both worm species have shown that gut motility is altered in animals infected with parasites compared to uninfected controls, perhaps this may account for some of the pH differences in the lower GI tract segments (Gregory et al. 1985, Bueno et al. 1982, Poppi et al. 1985). Lambs infected with *T. colubriformis* showed GI motility disturbances with an increase in the frequency of MMC (Gregory et al. 1985). Similar findings in sheep infected with *H.contortus* showed increased antral contraction and an increase in the number of duodenal MMC per day during larvae development (Bueno et al. 1982). This study also noted an increase in duodenal digesta flow which was found to be due to ionic permeability changes in the GI mucosa. In another study lambs infected with *T. colubriformis* had excessive bile in the duodenum, suggesting motility was impaired (Poppi et al. 1985).

Although *T. colubriformis* establishes in the proximal 3 meters of the small intestine initially, as an immune response is mounted worms could be expelled to inhabit

further down the small intestine in the ileum and even the caecum (Wagland et al. 1996). Perhaps nematode ES products and the immune response may account for some of these pH differences.

There are numerous studies reporting a rise in abomasal pH with worm infections (Ross et al. 1968, Murray et al. 1970, Malczewski 1971, Simpson et al. 1997, Hertzberg et al. 2000). Although pH tended to be slightly raised in the abomasal digesta of lambs infected with *H. contortus* compared with that of those infected with *T. colubriformis* and also the controls, this was not significant. It is possible that pH increased early after infection but returned to normal by time of slaughter. In this experiment lambs infected with *H. contortus* and *T. colubriformis* were slaughtered 35 and 28 days post-infection, respectively. Calves infected with *Ostertagia ostertagi* were found to have a pH of 6.42 between 21-30 days post infection (Murray et al. 1970). Secretory and motor disturbances in both the abomasum and duodenum with infection of *H. contortus* has been shown to result in substantial rise in abomasal pH (to almost 7) at the beginning of about 4 days after infection and continues for about a week when the L4 are moving from the gastric pits to the surface of the mucosa (Malczewski 1971).

Animals did not display any clinical signs of parasitism so it can only be hypothesised that worm burden were not high enough to exhibit an effect on acid production and secretion.

5.5.2 Condensed tannin

The concentration of the free, protein-bound and fibre-bound CT were determined and the total CT calculated for digesta from GI tract segments of lambs that had previously been fed a diet of calliandra. It is of interest to see if either species of worm or no worm had any significant effect on CT concentrations in the GI tract segments, and if pH has any relationship with the different CT fractions in the different segments.

Knowing the amount of each fraction of CT that is present in each GI segment is of major importance with respect to potential anthelmintic effects, if in fact CT is to be

available to complex with and have an effect on GI nematodes or on protein and carbohydrate metabolism.

5.5.3 Concentration of condensed tannin in calliandra

The calliandra used as the feed in this experiment contained a high level of total CT; 11.95 % DM (Table 5.12). Values from other studies have reported values of 7.5, 5.8, 6.1, 11.6, 11.7 and 19.4 % DM (Ahn et al. 1989, Jackson et al. 1996a, Perez-Maldonado and Norton 1996, Balogun et al. 1998). The total value obtained from the analysis of CT from a plant does however depend on other factors such area grown, time of year, soil fertility and the type of tannin that is used to standardise the assay.

5.5.4 Effect of GI nematodes on concentration of CT fractions and total CT in GI segments

There were no significant effects ($P < 0.05$) due to the presence of either of the two worm species, *T. colubriformis* or *H. contortus* or with no worms on CT concentrations for the any of the fractions of CT in any of the GI segments.

5.5.5 Effect of GI segment on concentration of CT

There was a significant effect of GI segment on the concentration of all fractions of CT and total CT for overall means (pooled for worm species and no worms), with $P < 0.001$ for all CT fractions and total CT (Table 5.9).

The concentration of each individual fraction as well as the total CT concentration changed along the GI tract. It may have been expected that the total CT might have stayed the same and the binding and releasing from protein and fibre would be reciprocal. This was, however, not the case in this study.

All fractions as well as total CT did change significantly along the GI tract as influenced by segments.

5.5.6 Interaction between CT and pH in the GI tract

The strength of the tannin-protein complex depends on characteristics and chemistry of both the tannin and the protein (molecular weight, tertiary structure, isoelectric point, and compatibility of binding sites). These interactions are also influenced by

the reaction conditions (temperature, pH, and tannin: protein ratios). These tannin complexes are reversible and can be disrupted by detergents or pH (Jones and Mangan 1977, Hagerman and Butler 1978). Proteins are precipitated by CT most efficiently at pH values near their isoelectric point where the protein-protein electrostatic repulsion is minimal (Hagerman and Butler 1981). The isoelectric point for purified plant protein is about 5.5 (Jones and Lyttleton 1972).

In ruminants most of the soluble proteins are degraded rapidly in the rumen and almost half of the N is converted to ammonia. If an animal ingests a plant containing CT then masticating will release plant protein and then the CT may be available to complex with these plant proteins as well as with rumen microbes. Barry et al. (1986) confirmed that ruminal degradation of protein was decreased due to CT in *Lotus pedunculatus* altering the protein or protein complexing. Growth inhibition and CT binding with rumen microbes has been demonstrated (Nelson et al. 1997, Bento et al. 2005). Barahona et al. (2006) found that CT inhibited fibrolytic enzyme activity. A study by McSweeney et al. (2001) found that feeding a 30% calliandra diet to sheep resulted in a reduction in the population of fibre-degrading rumen bacteria, *Fibrobacter succinogens* and *Ruminococcus spp*. The effect on bacteria appear to be quite different at low and high CT levels, with low CT levels of 0.14 mg/mL increasing microbial yield (Mbugua et al. 2005) which are at levels far below that recorded in the rumen in this study at 19g/L. In a study by Molan et al. (2001) that high levels of CT in the rumen resulted in a lower bacterial yield.

There was a significant quadratic relationship between pH and free CT with the lower the pH the higher the free CT concentration (Figure 5.8). There was also a significant quadratic relationship between pH and total CT with the lower the pH the higher the total CT concentration (Figure 5.9). No significant relationships were found to exist between protein or fibre-bound CT and pH. The total CT and pH relationship is due to the free CT.

The mean rumen pH value for the calliandra fed lambs in this study was 6.7 (range of pH 6.5-6.9), with the majority at 51% of the CT that was able to be detected in the assay in the protein-bound form, and 29% and 20 % as free and fibre-bound CT,

respectively. This mean pH is in the upper of the normal range for the rumen of pH 5.5-7. If CT binds to these proteins then the protein will be unavailable for the microbes use as an energy source. This agrees with other studies of Jones and Mangan (1977) who found that at rumen pH of 6.5, CT was tightly bound to protein with the most stable CT-protein complexes occurred between pH 4-7, which is in the range found in this experiment. A study by Perez-Maldonado et al. (1995) disagrees with this range and reports that the most stable CT protein complexes occurred at pH 3.5-5.5, however, when different inorganic ions were added to the digesta, this stable protein-CT complex could also be stable at pH 6-7. Diaz-Hernandez et al. (1997) conducted *in vitro* work with CT protein complexes and found that protein CT complexes only occurred at pH values between 3 and 7.

In the acidic conditions that occur in the abomasum and duodenum the CT may dissociate from the protein and be available for use by the animal to be digested and absorbed. The decrease in pH of the abomasum may, therefore, induce the CT previously bound to protein in the rumen to be released. In this study the pH went from pH 6.7 in the rumen with 51% protein-bound CT to a pH of 3.3 (range of pH 2.0-4.8) in the abomasum and only 2% now as protein-bound CT. In the abomasum 95% of CT was in the free CT fraction. The percent of CT able to be detected that was protein and fibre-bound were 2% and 3%, respectively, for the abomasum. This is in close agreement with Jones and Mangan (1977) who reported that at pH 1-3 that 95% of CT was dissociated.

The pH in the duodenum was found to be 4.8 (range of pH 3.6-6.3), with only 7 % CT still protein-bound and unavailable to the animal. Fifty five percent of the CT was in the free CT fraction and 38 % was in the fibre-bound CT fraction. This 55% free CT would now be digested and made available for use by the animal.

The pH in the ileum was 7.6 (range of 7.1-7.9), which was the highest mean recorded for any segment. There was 19, 31 and 50% as free, protein-bound and fibre-bound CT, respectively in this segment. This pH is in between the values quoted by Jones and Mangan (1977) of pH 4-7 having stable protein-CT complexes and pH between

8-8.5 that 30 % of CT was dissociated. As this is in between these values it is reasonable to expect the 19 and 31 % of CT are free and protein-bound.

The lower GI tract segments, caecum and colon recorded pH values of 6.7 (range of pH 6.98-7.34), and 7.3 (range of 7.05-7.51), respectively, and had similar values for the percent detected of the three fraction of CT. The caecum and colon had 6% and 3% free CT, 43% and 41% for protein-bound CT and the highest proportions of 49% and 53% fibre-bound CT, respectively.

The faeces, although collected at a different time interval, were also in agreement with the values for lower GI tract CT proportions (Figures 5.15 and 5.16).
From the above it could be suggested that there is still a reasonable proportion of protein-bound CT in these lower segments and that N would be excreted and not utilized by the animal. This is in agreement with other studies who reported that animals fed calliandra, that protein was expelled in the faeces (Merkel et al. 1999, Widiawati 2002, Cresswell 2007). The caecum and rumen both had a mean pH of 6.7 for calliandra fed lambs, however, the percent of protein-bound CT as a percent of total detectable by the assay were 43% and 51%, respectively. Although the pH was the same this suggests that other factors are involved in influencing the CT protein complexing, perhaps ionic factors as reported by Perez-Maldonado et al. (1995), ES products from the nematodes or other secretory products present in those GI tract segments. In the rumen this was the greatest percent complexed but in the caecum the fibre-bound percentage was higher at 50 % of total CT detected.

5.5.7 How are the CT fractions interacting with the GI nematodes?

There have been numerous studies to date reporting both the direct and indirect anthelmintic *in vivo* effects of feeding sheep and other ruminants on plants that contain CT or drenching with CT extracts (Table A2.2). These effects range from no effect to reductions in FEC, fecundity and worm burden and variable results for LWG. Using CT extracts in *in vitro* assays such as EHA, LFIA, LEA and LMIA (Table 2.11) have also demonstrated effects from no effect to inhibition in the particular assay.

From the results of CT analysis it can be seen that the majority of the protein-bound CT was found in the rumen and the lower GI tract segments, ileum, caecum and colon where the pH ranged from 6.7-7.6. It can be assumed then that in these segments the CT may be in favourable conditions to complex with the nematode adult or larvae cuticle or the eggs. Seeing the adult *H. contortus* and *T. colubriformis* inhabit the abomasum and duodenum respectively, it could be assumed that as these segments contain mostly free CT and very little protein-bound CT complexes that the effect is not on the adult cuticle as such but may be more indirect.

The *H. contortus* which inhabits the abomasum ingests and bathes in sheep blood and might receive a very small amount of absorbed phenolics via this route, however, the CT effect would have to be from contact or complexing with the cuticle (consisting mainly of proteins and lipids) and possibly being absorbed through the cuticle as very little (3% of total detectable CT) was found in this segment. Other possibilities are that CT may be affecting larvae or eggs. Exsheathment of *H. contortus* occurs in the rumen where stable protein-CT complexes form. Studies by Brunet et al. (2008), have shown that exposure of exsheathed L3 could not penetrate the fundic explants at a CT dose of 1200 μg/mL. This was reversible when treated with PVPP. This suggests that there is an interaction between the larvae and the CT in the abomasum but the effect on the worms is not known. You might expect that this would then result in less adults and then less egg counts. In the study by Cresswell (2007) there were no significant differences in *H. contortus* worm burdens for the calliandra fed lambs compared to lambs fed a non-tannin diet of lucerne pellets. It is possible that the CT may have an effect on the eggs, which are permeable to chemicals, during the eggs journey down the lower GI tract where there the protein-bound CT percentages are high.

T. colubriformis which are duodenal grazers, would ingest free, protein-bound and fibre-bound CT in the duodenum. The digesta was found to contain 55% free CT, 38% fibre-bound CT and very little protein-bound CT (7%). This suggests that very little CT would be complexing with the nematode cuticle and so the only possibility of CT exerting an anthelmintic effect would be due to absorption by the cuticle or

from ingestion of CT. The eggs would have the same exposure along the GI tract as the *H. contortus* eggs, however, the eggs of the *T. colubriformis,* are not permeable.

The study by Cresswell (2007) was a separate study to this thesis, which collected different data but shared the same experimental lambs and so data for effects of CT on nematodes is matched to this thesis. In this study Cresswell (2007) found that egg production decreased by 64-84% and 24-68% in *H. contortus* and *T. colubriformis* respectively in these lambs on the diet of calliandra. This concludes that the CT diet did have an effect on the nematodes, however, is still unclear how that effect was done. In a staining experiment Cresswell (2007) found CT on the outside of adult intact *H. contortus* and *T. colubriformis*, however, this was mainly in adherent digesta. There was CT stained in the pharynx and intestines of *H. contortus.* There was also some staining around the female vulval flap and one female found to have some staining on the surface cuticle striae. In two females in this study faint staining was found in the edges of the oespohagus. In sections from the GI tract, stained for CT, Cresswell (2007) found the lumen of the rumen only was stained but in all other segments, CT was found in the lamina propria, especially in the abomasum.

In another study on *H. contortus* exposed to tannin-rich plants there were structural changes in the cuticle (longitudinal and transverse folds and thicker cuticle ridges) and buccal areas as detected by electron microscopy suggesting an effect on worm motility, reproduction and nutrition (Martinez-Ortiz-de-Montellano et al. 2013).

5.5.8 Significant relationships between CT fractions and also total CT

Condensed tannin can exist as protein-bound, free and fibre-bound in a plant, with the binding of CT to protein and fibre interchangeable and reversible along the GI tract. The CT- complexes binding and dissociation is influenced by pH, presence of plant and animal proteins, microbes and enzymes, as well as the presence of bile secretions in the duodenum.

There were significant relationships between free and total CT, protein-bound CT and both free and total CT, as well as between total CT and both protein-bound and free CT. This highlights the fact that only some CT fractions are interchangeable and related.

5.5.9 Accounting for total CT along the GI tract

Not all of the CT in the ingested feed was able to be detected in the digesta samples from the GI tract segments assayed. Only 39% was detected in the rumen digesta, however, once in the abomasum, the highest amount was detected at 96%. As the digesta moved to the duodenum only 24% was detected. The lower GI tract segments were similar at 47% for the ileum and 49% for both the caecum and colon. This does not show any pattern of progressive loss along the GI tract, but perhaps very segment dependant. pH is not the only factor playing a role here as the highest percentage detected is at pH 3.3 and the lowest at pH 4.8. In digesta at similar pH in the lower GI tract the percent detected is similar, but at pH of the rumen which is the same as the caecum the percent detected are 39 and 49%, respectively. Perez-Maldonado et al. (1995) also reported that with feeding lambs calliandra, 76% of free CT was lost after entering the rumen and post abomasal only 57% of the free CT was absorbed. For protein bound CT Perez-Maldonado et al. (1995) found a net gain of 83% protein-bound CT across the rumen and that fibre-bound CT progressive decreased from rumen to faeces.

There appears to be losses of detectable CT and then reappearance in different GI tract segments. Whether this is an actual loss or just undetectable with the assay or whether there is metabolism and absorption remains inconclusive. Many studies report similar findings with regard to digesta and faecal samples.

The work of Perez-Maldonado (1994) and Perez-Maldonado and Norton (1996) demonstrated that CT metabolites may be absorbed into the blood stream and also excreted in the urine and faeces. This is also supported by ^{14}C studies by Terrill et al. (1994) who after feeding ^{14}C incorporated plants, the radioisotopes were detected in the urine and liver, although no ^{14}C was detected in the blood. Terrill et al. (1994) found in ^{14}C labelled CT ingested, 92.5% was recovered, with 88.8% being in the digesta, 2.9% in gut tissue, 0.14% in faeces, 0.63% in urine and 0.0008% in liver. There was ^{14}C label detected in post-abomasal gut tissue to the end of the small intestine and then disappeared with smaller amounts detected in the proximal colon and caecum, perhaps this binding to the gut accounts for the losses in the GI tract in other studies (Terrill et al. 1994, Perez-Maldonado and Norton 1996). This is in

agreement with Cresswell (2007), who found in the CT stained gut tissue sections where the lumen of the rumen was stained as well as the lamina propria in all other segments, especially in the abomasum.

Table 5.15 Summary of detection and/or losses of CT in digesta and faeces.

	% of CT added or ingested			
	1	2	3	4
Rumen	82.3	56.6	50.5	39
Abomasum	45.5	95.4	52.9	96
Duodenum	69.8	-	-	24
Ileum	45.7	35.3	-	24
Caecum	-	-	-	49
Colon	-	-	-	49
Faeces	-	48.1	17.7	46

1. Recovery of 15-20 mg condensed tannin (CT) added to 15 mL digesta from rumen, abomasum, duodenum or ileum of a sheep fed on perennial ryegrass (*Lolium perenne)*/ white clover (*Trifolium repens*) pasture, from Terrill et al. (1994) analysed using the method from Terrill et al. (1992b).
2. Recovery of condensed tannin (% of total CT in feed) from rumen, abomasal and ileal digesta and faeces of sheep fed *Lotus pedunculatus* containing 5.7% condensed tannin*,* adapted from (Terrill et al. 1994), analysis using the method from (Terrill et al. 1992b).
3. Recovery of condensed tannin (% of total CT in feed) from digesta from rumen, abomasum, urine and faeces of sheep and goats fed *Calliandra calothyrsus*/pangola grass (*Digitaria decumbens*) hay diet containing 2.25% condensed tannin, adapted from (Perez-Maldonado and Norton 1996), analysis using method of (Perez-Maldonado 1994).
4. Recovery of condensed tannin (% of total CT in feed) from digesta from rumen, abomasum, and faeces of lambs fed *Calliandra calothyrsus* diet containing 11.95% condensed tannin. Results from this experimental work, analysis using the modified method from (Terrill et al. 1992b).

Condensed tannin recovery from feed spiked with CT was close to 100% (Terrill et al. 1994), however, the recovery of CT added to digesta was < 100%. This suggests that the CT has undergone a conformational change so that it is no longer able to be detected by this assay, or there is interference by a substance in the digesta. Perez-Maldonado and Norton (1996) investigated the metabolism of CT in the GI tract of sheep, it was found that by using the modified *n*-butanol-HCl method of Perez-Maldonado (1994) for determining the protein-bound fraction of CT, that more of the CT could be dissociated from the complex as compared to the method of Terrill et al. (1992b).

The results from this experiment still leaves some debate as to the metabolism of CT in the digesta and how the digesta is affecting the assay for CT and furthermore if the assays are sufficient to detect all of the CT fractions.

5.6 Conclusion

From this study it can be concluded that digesta pH may be influenced by diet and nematodes in some of the GI segments. There was a significant diet and worm interaction in the abomasum only with pH being higher both in the digesta from calliandra fed lambs that were infected with worms. Diet had a significant effect on pH values in the abomasum, with the pH in the lower GI segments, ileum, caecum and colon being consistently higher in digesta from lambs fed the CT containing calliandra diet. The presence of nematode infections significantly affected pH values in the ileum, caecum and colon with pH values being lower in the digesta from the lambs infected with either of the worm species compared to the uninfected controls in these three lower GI tract segments.

With respect to concentration of total CT and the three CT fractions along the GI tract, the presence of worms had no effect in any of the GI segments but there was a significant effect of GI segment on the concentration of all fractions of CT and total CT for overall means. There was a significant quadratic relationship between pH and free CT with the lower the pH the higher the free CT concentration. There was also a significant quadratic relationship between pH and total CT with the lower the pH the higher the total CT concentration.

It was found that not all the CT that was in the ingested feed was able to be detected in the digesta samples from the GI tract segments assayed with losses of detectable CT and then reappearance in different GI tract segments. The results from this experiment still leaves some debate as to the metabolism of CT in the digesta and how the digesta is affecting the assay for CT and, furthermore, if the assays are sufficient to measure all CT fractions.

CHAPTER 6 EXTRACTION AND PURIFICATION OF RUBISCO FROM PLANT TISSUE

6.1 Introduction

The leaf protein, Ribulose-1, 5-*bis*phosphate carboxylase (EC 4.1.1.39: Rubisco) is the most abundant protein on earth and accounts for approximately 30-50 % of total plant protein. Rubisco is involved in the primary catalytic step in photosynthetic CO_2 fixation and is characterised by eight large subunits with a molecular weight of 54 000 and eight small subunits with a molecular weight of 16 000 (Kawashima and Wildman 1970), making them readily detectable by the method of SDS polyacrylamide gel electrophoresis (PAGE).

Purified Rubisco was required for subsequent experimental work involving *in vitro* complexing of protein and fibre with CT. An important characteristic with regard to complexing CT is the isoelectric point of about pH 4.3-5.5 (Pon 1967). Proteins are precipitated by CT most efficiently at pH values near their isoelectric point where the protein-protein electrostatic repulsion is minimal (Hagerman and Butler 1981).

The purchase of commercially available purified plant protein proved to be too expensive for the experimental work in the quantities required. It was therefore, decided to extract Rubisco from *Spinacia oleracia* (spinach) leaves, however, would be preferable to obtain purified Rubisco for the complexing studies with CT from the same plant that the CT was extracted from, which is calliandra.

6.2 Materials and Methods

6.2.1 Spinach

Baby spinach leaves were weighed and deveined before extraction.

6.2.3 Extraction and purification

Rubisco was extracted from 2.5 kg of plant tissue using the rapid method of Hall and Tolbert (1978). The spinach was extracted in five batches of 500 g each. Each batch was divided into two x 250 g lots for blending as 500 g could not be accommodated

in the 1 litre waring blender, after which the extracts were combined and treated as one. An extra magnesium chloride ($MgCl_2$) precipitation was added as one-third of the protein was still left in the supernatant after the first precipitation. The enzyme was applied to and eluted from a diethylaminoethyl (DEAE) cellulose column (5.2 x 30 cm) as described by Hall and Tolbert (1978). The fractions containing protein were pooled and brought to 50% saturation with ammonium sulphate (30.5g/100 mL), spun at 16 000g for 30 min and the pellet dissolved in 40-50 mL buffer. This was desalted by gel filtration through a sephadex G-25 (medium) column (3 x 37 cm, void volume = 120 mL and column volume = 350 mL) pre-equilibrated with grinding buffer. The desalted samples were freeze-dried (Dynavac, Dynavac Engineering Pty Ltd, Sydney) for 42 h. The freeze-dried Rubisco was stored desiccated at –20°C (Keys and Parry 1990).

6.2.4 Protein

Spinach leaves were analysed for CP content using the Kjeldahl method (A.O.A.C, 1955). Fractions collected during extraction and purification containing protein were identified by measuring absorbance at 595 nm (Lambda EZ210 UV/visible spectrophotometer, Perkin Elmer, Connecticut, USA). Protein content was measured using the Bradford Assay (Bradford 1976) to determine the µg protein/mg of sample. The assay was calibrated with BSA 0-10µg/mL. Bradford's reagent and BSA micro standards (1 mg/mL) were purchased from Sigma-Aldrich, Australia.

6.2.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Purified spinach leaf extract was analysed by SDS PAGE to determine purified Rubisco content. A commercially available SDS PAGE kit was used (Life Therapeutics, Australia). Purified Rubisco, commercially available spinach Rubisco (Sigma-Aldrich, Australia) and a pre-stained protein ladder (~10-180kDa) (Quantum Scientific, Milton, Australia) were all run on a Pre cast 12% 1mm polyacrylamide electrophoresis gel with Tris-HEPES-SDS buffer at 150v for 35 min using a Mini Protean 3 apparatus. The gel was washed for 10 min in deionised water and stained overnight in Coomassie stain (50% methanol, 10% acetic acid and 0.2% Coomassie blue R-250 in deionised water). After destaining for 7 h in 6% acetic acid, the gel

was put onto a drying frame with a gel soaking/drying solution containing 40% methanol, 10% glycerol and 7.5% acetic acid.

6.2.6 Calliandra

Two to three young calliandra leaves were collected from the end of each branch of calliandra trees after two months previously been harvested to 1m. The leaves were placed immediately in snap lock bags between dry ice and after 20-30 min placed in a -20° C freezer. To account for the CT content of the leaves, 100 g of leaves were added to 100 g of PVPP, 8 g of casein and 800 mL of grinding buffer and then extracted as described by Hall and Tolbert (1978). Gray (1978) showed that at concentrations of 380-500 mg dry weight per mg CT that 90% of the CT was removed using insoluble PVPP.

6.3 Results

6.3.1 Dry matter and crude protein

The spinach leaf was found to contain 34% CP and to have a DM content of 5.86%.

6.3.2 Extraction and purification of Rubisco from baby spinach

Means of the five batches for total extraction and purification of leaves collected and protein yields of the various fractions during extraction and purification of Rubisco from spinach leaves can be seen in Table 6.1.

Table 6.1 Means ± standard error of the means of the five batches for total protein (g) and percent protein yield of the various fractions obtained during extraction and purification of spinach leaves.

Fraction	Total Protein (g)	Yield (%)
Crude extract	5.40 ± 0.36	100
PEG cut	5.10 ± 0.25	92.6
Protein pellet redissolved	2.73 ± 0.22	50.0
After DEAE	0.98 ± 0.03	18.5
After G-25 desalting	1.03 ± 0.09	18.5

The extraction and purification of Rubisco from spinach leaves resulted in a mean protein yield of 18.5% and a mean total of 1 g of protein per 500 g fresh weight of spinach leaves. A total of 2568.9 mg of Rubisco was extracted from the 2.5 kg fresh weight of spinach leaves (five batches) which contained 0.65 mg of protein/mg of freeze dried sample.

The SDS PAGE run with purified Rubisco from spinach leaves, commercial Rubisco and the standard marker is shown in Plate 6.1

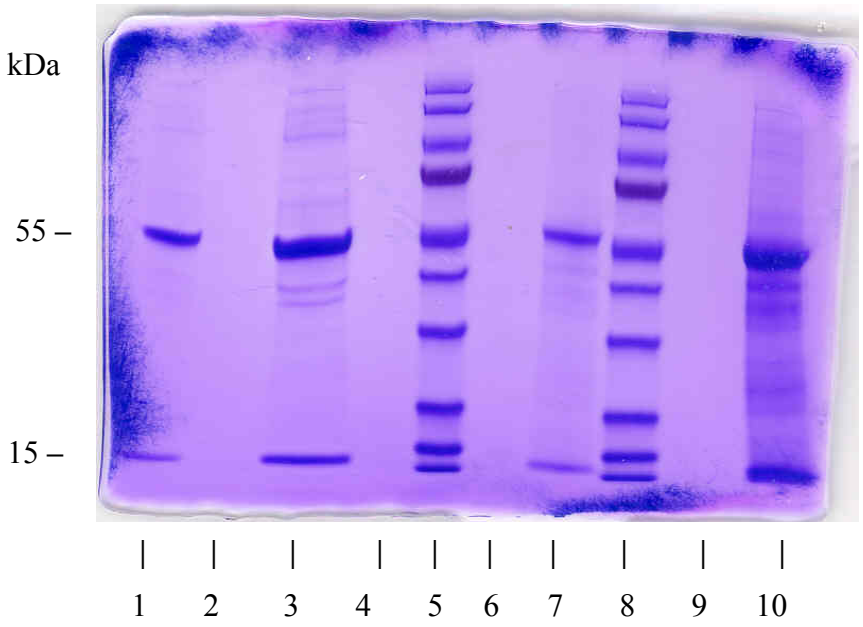


Plate 6.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis of spinach extracts. Samples were electrophoresed on 12% Sodium dodecyl sulphate polyacrylamide gel electrophoresis and stained with Coomassie R-250. Lane 1, Purified spinach leaf extract (2µg); Lane 3, Purified spinach leaf extract (10µg); Lane 5, Standard marker (10 µL); Lane 7, Sigma Rubisco (2 µg); Lane 8, Standard marker (10 µL); Lane 10, Sigma Rubisco (10 µg).

6.3.3 Extraction and purification of Rubisco from 100 g of young calliandra leaves.

Protein and CT concentrations of the various fractions during extraction and purification of Rubisco from calliandra can be seen in Table 6.2.

Table 6.2 Protein concentration (mg/mL), total protein (mg), condensed tannin (CT) concentration (mg/mL), total free condensed tannin (mg) and protein to condensed tannin ratio of the various fractions obtained during extraction of calliandra.

Fraction	Protein (mg/mL)	Total protein (mg)	CT (mg/mL)	Total free CT (mg)	Protein:CT
Crude	0.12	10.49	0.13	39.04	1 : 3.7
After $MgCl_2$ cut	0.03	8.51	0.08	27.65	1 : 3.3
Protein pellet	0.40	2.00	0.03	1.72	1.1 : 1

The extraction technique was not sufficient enough to remove the entire CT from the calliandra extract (Table 6.2). The crude calliandra extract contained only a small amount of Rubisco compared to the crude spinach leaf extracts, with 10.49 mg from 100 g of calliandra and 5.40 g from 500 g of spinach.

6.4 Discussion

Extraction and purification of Rubisco from spinach leaves was successful with a yield of approximately 1 g/kg from the fresh spinach leaves. This is slightly lower than reports from other studies of yields of 1.5 g/kg Rubisco from white and red clover leaves and 1.6-2 g/kg from fresh spinach leaves (Jones and Lyttleton 1972, Hall and Tolbert 1978). The SDS PAGE confirmed that the protein extracted from the spinach leaves was Rubisco, and hence suitable as purified Rubisco to use in subsequent experimental work in chapter 7.

During the extraction of Rubisco from calliandra it appeared that the CT was mostly removed, however, so too was protein from the extract. If calliandra contains about 3.7 g CT /100g fresh weight, then PVPP and casein removed 99% of the free CT. Calliandra consists of 24% CP (DM) then 100 g fresh weight should contain 8.88 g protein. The actual amount of protein extracted was 1.9 mg which is only 0.002% of the potentially extractable protein. The protein was most likely to be complexed with the CT and spun out with the PVPP pellet.

Other workers have tried various combinations of PVPP, casein, BSA, Dowex-1, porous polystyrene and other ion and anion exchangers, with varying degrees of

success of removing the CT (Loomis et al. 1979, Gray 1978, Magel et al. 2001). Spinach Rubisco is easily extracted and commonly used in experimental work so it was decided to proceed with the purified Rubsico from spinach leaves for chapter 7.

6.5 Conclusion

Rubisco was successfully extracted and purified from spinach leaves, yielding 1 g/kg of Rubisco, however, attempts to extract and purify Rubisco from calliandra were unsuccessful due to not being able to remove CT from the extract without considerable protein losses.

CHAPTER 7 *IN VITRO* COMPLEXES OF CONDENSED TANNIN

7.1 Introduction

Condensed tannins are naturally occurring water-soluble plant polyphenols, which have the ability to bind and precipitate proteins and can also form complexes with minerals and carbohydrates, including cellulose, hemicellulose and pectin (Price and Butler 1977). Condensed tannins can exist as free (unbound), protein-bound and fibre-bound fractions in plants as well as in digesta in the GI tract.

Chapter 5 investigated the interaction between pH and CT binding and dissociation in lamb digesta in different segments along the GI tract, as well as the influence of nematodes and diets containing either high CT or no CT on pH. The *in vitro* experimental work in this chapter was designed to compliment chapter 5 and investigate CT complexing with protein and fibre.

Results from chapter 5 showed that there were correlations between pH and free CT and pH and total CT. In the analysis of the fractions of CT, however, there was some variability. The CT appeared to be lost and reappear along the GI tract in different segments, which could be due to the digesta interfering with the assay or something in the digesta rendering the CT undetectable.

The physiologically possible pH values in the GI tract range from 1-9. The range of pH values recorded in chapter 5 were from 1.95 in the abomasum to 7.89 in the ileum. Transit times in different segments may range from 0.5-24 h in which there is complexing and dissociation of CT between protein and fibre.

The aim of this experiment was to determine pH range and complexing times at which protein and fibre complexes with and dissociates from calliandra CT *in vitro*.

It was hypothesised that:

i. *in vitro binding of condensed tannin with and dissociation from protein and fibre is pH dependant,*
ii. *binding of condensed tannin with protein is greater in acidic pH range of 4-7,*
iii. *binding of CT with protein is greater at complexing times greater than 8 h.*

7.2 Materials and Methods

7.2.1 Condensed tannin, cellulose and Rubisco

This *in vitro* complexing study used purified CT extracted from calliandra (chapter 3) and purified Rubisco extracted from spinach (chapter 6) and commercial high purity cellulose powder (Sigma-Aldrich fibres, long, Sigma-Aldrich, Australia).

7.2.1.1 Condensed tannin, cellulose and Rubisco ratios

For the experiments to simulate complexing in the digesta *in vivo*, similar concentrations and ratios of CT, protein and fibre found in the digesta was used. Data from analysis of calliandra samples from previous work of Widiawati (2002), Cresswell (2007), Perez-Maldonado and Norton (1996), Terrill et al. (1992b), Jackson et al. (1996a) are shown in Table 7.1.

Table 7.1 Concentration of condensed tannin (CT), crude protein (CP) and acid detergent fibre (ADF) in feed [% dry matter (DM)] and rumen digesta (mg/mL).

	% DM (feed)	CT (mg/mL) rumen digesta	CT (mg/mL) rumen digesta	CT (mg/mL) rumen digesta
CT	6	30	19	2.2
CP	21	102	-	11.7
ADF	31	155	-	28.3
Reference	1	2	3	4

1. (Widiawati 2002); [CT] in calliandra grown in North Queensland reported as 5.78% (Jackson et al. 1996a); 2. (Cresswell et al. 2004); Sheep in which anthelmintic effect was found were fed diet of 100% calliandra at 1.2-1.5kg per day. On slaughter days experiment rumen volumes were measured and ranged from 1.6-4 L, means of 2.9 L.; 3. (Cresswell 2007); fed diet of 100% calliandra; 4. (Perez-Maldonado and Norton 1996); fed 30% calliandra with mixed diet composition. Rumen volume reported at 7.9 L.

Similar ratios and concentrations in lambs as reported by Cresswell (2007) were used in chapter 5 for comparisons.

The concentration of CT in sheep digesta from work of Perez-Maldonado and Norton (1996) was 2.2 mg/mL and also from Terrill et al. (1992b) used 1-2 mg/mL, both these authors used 1 mL in their reactions. Similar amounts were used in these assays as they were able to detect these concentrations within the limits of their assays. It was decided to use a ratio of CT:cellulose:Rubisco of 1:5.3:3.6, which is 2 mg/mL of CT, 7.2 mg/mL Rubisco and 10.6 mg/mL cellulose in a 2 mL reaction, buffered with 50 mM of 0.1% ascorbic acid. Ionic strength (0.05-0.50) and ascorbic acid strength (0.10-1.0%) has been shown to have no effect on release of protein or the degradation at either 0 or 39°C (Jones and Mangan 1977).

7.2.2 pH and complexing time

The pH values and complexing times used in this experiment are shown in Table 7.2.

Table 7.2 pH and complexing times (h) for *in vitro* assays.

pH	Complexing Time (hours)			
1	0.5	3	8	24
1.5	0.5	3	8	24
2	0.5	3	8	24
2.5	0.5	3	8	24
3	0.5	3	8	24
4.5	0.5	3	8	24
4	0.5	3	8	24
5	0.5	3	8	24
6	0.5	3	8	24
7	0.5	3	8	24
8	0.5	3	8	24
9	0.5	3	8	24

7.2.3 Samples and analysis

Five replicates for each sample were analysed for free CT, fibre-bound CT and protein-bound CT. Rubisco, CT and cellulose were weighed out the day before the complexing assay and CT and Rubisco were stored at 4 °C. Immediately before the complexing, the CT was dissolved in deionised water at a concentration of 20 mg/mL, and the Rubisco and cellulose dissolved in the appropriate buffers of pH 1-9 at a concentration of 10.29 mg/mL and 26.5 mg/mL, respectively. Details for buffer solutions are shown in Table 7.3.

Table 7.3 Details of buffers used for each pH value.

pH at 25ºC	pH at 39ºC	Buffer	Formula	pKa	Mwt	Concentration of acid (M)	dpK_a/dT	Volume of 50 mM acid added (g/L)
1.0616	1.0	Orthophosphoric acid	H_3PO_4	2.15	98	15	+0.0044	3.35mL
1.5616	1.5	Orthophosphoric acid	H_3PO_4	2.15	98	15	+0.0044	3.35mL
2.0616	2.0	Orthophosphoric acid	H_3PO_4	2.15	98	15	+0.0044	3.35mL
2.5616	2.5	Orthophosphoric acid	H_3PO_4	2.15	98	15	+0.0044	3.35mL
3.0616	3.0	Orthophosphoric acid	H_3PO_4	2.15	98	15	+0.0044	3.35mL
3.9972	4.0	Acetic acid, glacial	CH_3COOH	4.76	98.15	18	-0.0002	2.8mL
4.4972	4.5	Acetic acid, glacial	CH_3COOH	4.76	98.15	18	-0.0002	2.8mL
4.9972	5.0	Acetic acid, glacial	CH_3COOH	4.76	98.15	18	-0.0002	2.8mL
5.9608	6.0	Sodium dihydrogen orthophosphate	$NaH_2PO_4.2H_2O$	7.21	156.01	-	-0.0028	7.8005g
6.9608	7.0	Sodium dihydrogen orthophosphate	$NaH_2PO_4.2H_2O$	7.21	156.01	-	-0.0028	7.8005g
7.9608	8.0	Sodium dihydrogen orthophosphate	$NaH_2PO_4.2H_2O$	7.21	156.01	-	-0.0028	7.8005g
8.6080	9.0	Tris (hydroxymethyl) methylamine	$NH_2C(CH_2OH)_3$	8.06	121.14	-	-0.028	1.2114g

Four hundred µL of cellulose, 700 µL of Rubisco, 100 µL of CT and 800 µL of the pH buffer were added to a 16 mL glass screw cap vial. The vial were mixed and incubated at 39ºC in a water bath for 0.5, 3, 8 or 24 h. The samples were analysed with the modified method of Terrill et al. (1992b), (chapter 4), with the following modification for the complexing assay. After the appropriate time the tubes were removed from the water bath and cooled to room temperature in an ice bath. The tubes were spun at 3000 rpm for 20 min, after which the supernatant was carefully poured off into 5 mL plastic vials. This supernatant contained the free-CT fraction. SDS solution (2 mL) was added to the pellet, vortexed and incubated in a boiling water bath for 45 minutes, after which they were removed and cooled to room temperature in an ice bath. The tubes were spun at 3000 rpm for 20 min and the supernatant poured into 5 mL plastic vials and identified as the protein-bound CT fraction. The pellet was kept for analysis of the fibre-bound CT fraction. All samples were stored refrigerated until analysis.

7.2.4 Recovery of spiked CT

Triplicate buffer samples as well as deionized water were spiked with 2 mg of CT to check recovery of the CT at each buffer pH. Results of the CT assays of lamb digesta in chapter 5 showed that along the GI tract at different pH values not all of the CT could be detected in the assays.

7.3 Statistical Analysis

Data were tabulated using Microsoft Office Excel 2003 (Microsoft Corporation, USA) and analysed using SPSS Version 13.0 for Windows (SPSS Inc., USA).

Data were subjected to the non-parametric Kruskal Wallis k sample test in SPSS, and where significance was found ($P < 0.05$) data were then analysed using Mann-Whitney test. Spearman's correlation in SPSS was used to explore the relationship between pH and fractions of CT and the coefficient reported with 99.9% confidence intervals.

7.4 Results

7.4.1 Complexing time

Results for the concentration of free, protein-bound and fibre-bound CT for pH values 1 to 9 and after 0.5, 3, 8 and 24 h of complexing are shown in Tables 7.4 -7.6.

Table 7.4 Means ± standard error of the means of the concentration (mg/mL) of free condensed tannin (CT), at pH 1-9 after 0.5, 3, 8 and 24 h of complexing (n=5).

pH	Free CT (mg/mL)			
	Complexing Time (hours)			
	0.5	3	8	24
1	1.20± 0.02	0.85 ± 0.05	0.78 ± 0.02	0.62 ± 0.03
1.5	3.05 ± 0.01	3.18 ± 0.03	3.05 ± 0.03	3.12 ± 0.02
2	3.14 ± 0.01	3.11 ± 0.03	3.32 ± 0.02	2.64 ± 0.10
2.5	2.00 ± 0.04	2.16 ± 0.03	2.07 ± 0.03	2.19 ± 0.03
3	1.07 ± 0.03	0.46 ± 0.01	0.57 ± 0.02	1.04 ± 0.02
4	1.25 ± 0.01	0.51 ± 0.01	0.57 ± 0.02	0.73 ± 0.02
4.5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
5	0.04 ± 0.00	0.06 ± 0.00	0.05 ± 0.00	0.04 ± 0.00
6	0.02 ± 0.00	0.04 ± 0.00	0.06 ± 0.00	0.04 ± 0.00
7	0.07 ± 0.00	0.08 ± 0.00	0.06 ± 0.00	0.06 ± 0.00
8	0.18 ± 0.00	0.12 ± 0.00	0.12 ± 0.00	0.09 ± 0.00
9	0.65 ± 0.02	0.16 ± 0.01	0.00 ± 0.00	0.01 ± 0.00

Table 7.5 Means ± standard error of the means of the means of the concentration (mg/mL) of protein-bound condensed tannin (CT), at pH 1-9 after 0.5, 3, 8 and 24 h of complexing (n=5).

	Protein-bound CT (mg/mL)			
pH	Complexing Time (hours)			
	0.5	3	8	24
1	1.16 ± 0.06	1.00 ± 0.05	1.16 ± 0.01	1.05 ± 0.02
1.5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2	0.01 ± 0.01	0.03 ± 0.01	0.05 ± 0.00	0.01 ± 0.00
2.5	0.33 ± 0.03	0.23 ± 0.01	0.30 ± 0.02	0.24 ± 0.01
3	1.07 ± 0.02	1.23 ± 0.02	1.17 ± 0.01	0.99 ± 0.02
4	1.11 ± 0.02	1.25 ± 0.01	1.14 ± 0.02	1.04 ± 0.02
4.5	1.39 ± 0.02	1.15 ± 0.00	1.13 ± 0.01	1.14 ± 0.02
5	1.38 ± 0.01	1.22 ± 0.03	1.24 ± 0.03	1.21 ± 0.03
6	1.27 ± 0.05	1.52 ± 0.04	1.40 ± 0.03	1.28 ± 0.02
7	1.39 ± 0.03	1.36 ± 0.01	1.36 ± 0.01	1.22 ± 0.02
8	1.40 ± 0.01	1.37 ± 0.02	1.41 ± 0.03	1.32 ± 0.02
9	1.03 ± 0.00	1.29 ± 0.00	1.17 ± 0.00	1.25 ± 0.00

Table 7.6 Means ± standard error of the means of the concentration (mg/mL) of fibre-bound condensed tannin (CT), at pH 1-9 after 0.5, 3, 8 and 24 h of complexing (n=5).

	Fibre-bound CT (mg/mL)			
pH	Complexing Time (hours)			
	0.5	3	8	24
1	0.16 ± 0.02	0.57 ± 0.06	0.35 ± 0.01	0.33 ± 0.03
1.5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2.5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
3	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
4	0.04 ± 0.00	0.03 ± 0.00	0.06 ± 0.00	0.07 ± 0.00
4.5	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
5	0.04 ± 0.00	0.04 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
6	0.03 ± 0.00	0.02 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
7	0.05 ± 0.00	0.03 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
8	0.04 ± 0.00	0.03 ± 0.00	0.05 ± 0.00	0.04 ± 0.00
9	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

There was no significant effect ($P = 0.911$) due to complex time on CT concentrations for the any of the fractions of CT, however, the fraction of CT did have a significant effect on CT concentrations ($P < 0.001$).

7.4.2 Condensed tannin fractions and pH

Data for concentration of each of the three fractions of CT at pH values 1 to 9 pooled for complexing time are presented in Table 7.7 and Figure 7.1.

Table 7.7 Means ± standard error of the means of concentration of free, protein-bound and fibre-bound condensed tannin (mg/mL) pooled for complexing time at pH 1-9 (n=20).

	Condensed Tannin (mg/mL)			
pH	Free	Protein bound	Fibre bound	P value
1	0.86a ± 0.03	1.09a ± 0.02	0.35b ± 0.02	0.018
1.5	3.11b,c ± 0.01	0.00b,c ± 0.00	0.00a ± 0.00	0.005
2	3.05b,c ± 0.04	0.03b,d ± 0.00	0.00a ± 0.00	0.006
2.5	2.11b ± 0.01	0.27b,e ± 0.01	0.00a ± 0.00	0.006
3	0.79a,e ± 0.03	1.12a,f ± 0.01	0.04a,c ± 0.00	0.015
4	0.76a,e ± 0.03	1.14a,g ± 0.01	0.05a,c ± 0.00	0.015
4.5	0.00b,d,f ± 0.00	1.20a,h ± 0.01	0.01a,c,d ± 0.00	0.006
5	0.05b,d,f ± 0.00	1.26b,f,g,h ± 0.01	0.04a,c ± 0.00	0.021
6	0.04b,d,f ± 0.00	1.37b,h ± 0.02	0.04a,c ± 0.00	0.006
7	0.07b,d,f ± 0.00	1.33b,h ± 0.01	0.04a,c ± 0.00	0.007
8	0.13b,d,f ± 0.01	1.37b,h ± 0.02	0.04a,c ± 0.00	0.007
9	0.21b,d,e,f ± 0.06	1.19a,f,h ± 0.03	0.00a,e ± 0.00	0.012
P value	0.000	0.000	0.000	

Columns with different letters are significantly different ($P < 0.05$).

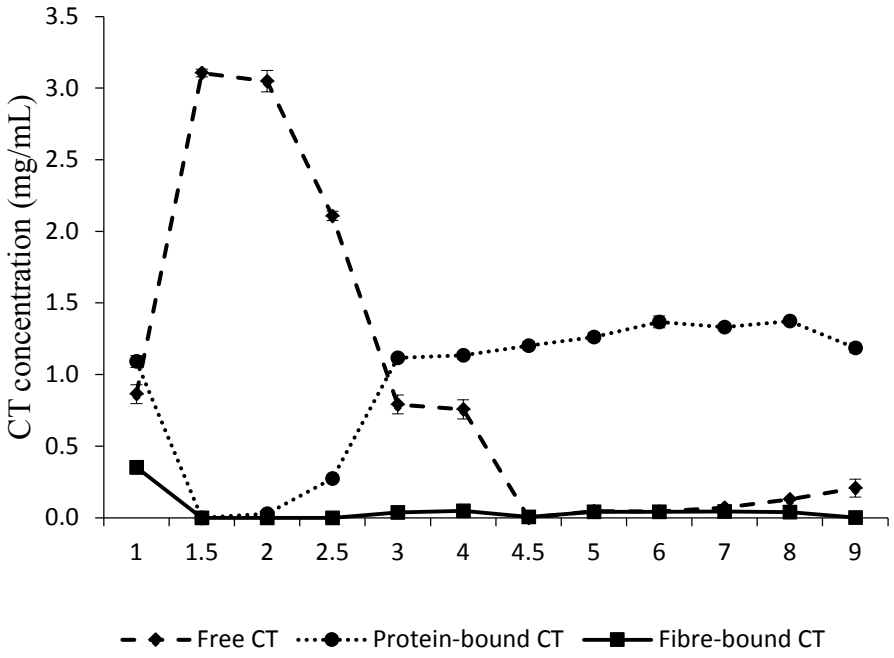


Figure 7.1 Means ± standard error of the means of concentration (mg/mL) of free, protein-bound and fibre-bound condensed tannin (CT) at pH 1-9.

There were significant differences in the concentration of CT between the free, protein-bound and fibre-bound fractions at each pH ($P < 0.05$) and between pH values for each fraction (Table 7.7).

There were significant differences in the concentration of free CT at pH 1 compared to samples at pH values 1.5 (P = 0.021), 2 (P = 0.021), 2.5 (P = 0.021), 4.5 (P = 0.014), 5 (P = 0.021), 6 (P = 0.021), 7 (P = 0.020), 8 (P = 0.021) and 9 (P = 0.043). There were significant differences in the concentration of free CT at pH 1.5 compared with samples at pH values 2.5 (P = 0.021), 3 (P = 0.021), 4 (P = 0.021), 4.5 (P = 0.014), 5 (P = 0.021), 6 (P = 0.021), 7 (P = 0.020), 8 (P = 0.021) and 9 (P = 0.021). At pH 2 and 2.5 there were significant differences in the concentration of free CT compared with samples at all other pH values (P = 0.021). There were significant differences in the concentration of free CT at pH 3 compared with samples at pH values 4.5 (P = 0.014), 5 (P = 0.021), 6 (P = 0.021), 7 (P = 0.020), 8 (P = 0.021). There were significant differences in the concentration of free CT at pH 4 compared with samples at pH values 4.5 (P = 0.014), 5 (P = 0.021), 6 (P = 0.021), 7 (P = 0.020), 8 (P = 0.021). There were significant differences in the concentration of free CT at pH 4.5 compared to samples at pH values 5 (P = 0.014), 6 (P = 0.014), 7 (P = 0.013), 8 (P = 0.014) and 9 (P = 0.047). There were significant differences in the concentration of free CT at pH 5 compared with samples at pH values 7 (P = 0.020) and 8 (P = 0.021). There were significant differences in the concentration of free CT at pH 6 compared with samples at pH values 7 (P = 0.020) and 8 (P = 0.021), except pH 9 (P = 1.000). There were significant differences in the concentration of free CT at pH 7 compared with samples at pH values 8 (P = 0.020), but not pH 9 (P = 1.000). There were no significant differences in the concentration of free CT at pH 8 compared with samples at pH 9 (P = 0.773).

There were significant differences in the concentration of protein-bound CT at pH 1 compared with pH values 1.5 (P = 0.014), 2 (P = 0.021), 2.5 (P = 0.021), 5 (P = 0.021), 6 (P = 0.0.021), 7 (P = 0.021), 8 (P = 0.021). There were significant differences in the concentration of protein-bound CT at pH 1.5 compared to samples at pH values 2-8 (P = 0.014) and 9 (P = 0.029). At pH 2 there were significant differences in the concentration of protein-bound CT compared with samples at pH values 2-9 (P = 0.021). At pH 2.5 there were significant differences in the concentration of protein-bound CT compared with samples at pH values 3-9 (P = 0.021). There were significant differences in the concentration of protein-bound CT at pH 3 compared with samples at pH values 6 (P = 0.021), 7 (P = 0.021) and 8 (P =

0.021). There were significant differences in the concentration of protein-bound CT at pH 4 compared with samples at pH values values 6 (P = 0.021), 7 (P = 0.043) and 8 (P = 0.021). There were no significant differences in the concentration of protein-bound CT at pH 4.5 compared with samples at pH values 5 (P = 0.248), 6 (P = 0.0.083), 7 (P = 0.149), 8 (P = 0.083) and 9 (P = 0.773). There were no significant differences in the concentration of protein-bound CT at pH 5 compared with samples at no significant differences in the concentration of protein-bound CT at pH 6 compared to samples at pH values 7 (P = 0.564), 8 (P = 0.773) and 9 (P = 0.083). There were no significant differences in the concentration of protein-bound CT at pH 7 compared with samples at pH values 8 (P = 0.248) and 9 (P = 0.083). There were significant differences in the concentration of protein-bound CT at pH 8 compared to samples at pH 9 (P = 0.021).

There were significant differences in the concentration of fibre-bound CT at pH 1 compared with samples at pH values 1.5 (P = 0.014), 2 (P = 0.014), 2.5 (P = 0.014), 3 (P = 0.021), 4 (P = 0.020), 4.5 (P = 0.021), 5 (P = 0.021), 6 (P = 0.021), 7 (P = 0.021), 8 (P = 0.021) and 9 (P = 0.018). There were significant differences in the concentration of fibre-bound CT at pH 1.5 compared with samples at pH values 3 (P = 0.014), 4 (P = 0.014), 4.5 (P = 0.013), 5 (P = 0.014), 6 (P = 0.014), 7 (P = 0.014), 8 (P = 0.014), except pH 2 (P = 1.000), 2.5 (P = 1.000) and 9 (P = 0.317). At pH 2 there were significant differences in the concentration of fibre-bound CT compared to samples at pH values 3 (P = 0.014), 4 (P = 0.014), 4.5 (P = 0.013), 5 (P = 0.014), 6 (P = 0.014), 7 (P = 0.014), 8 (P = 0.014). At pH 2.5 there were significant differences in the concentration of fibre-bound CT compared with samples at pH values 3 (P = 0.014), 4 (P = 0.014), 4.5 (P = 0.013), 5 (P = 0.014), 6 (P = 0.014), 7 (P = 0.014), 8 (P = 0.014). There were significant differences in the concentration of fibre-bound CT at pH 3 compared with samples at pH values 4.5 (P = 0.020) and 9 (P = 0.018), except pH 4 (P = 0.773), 5 (P = 1.000), 6 (P = 0.564), 7 (P = 0.773) and 8 (P = 0.773). There were significant differences in the concentration of protein-bound CT at pH 4 compared with samples at pH values 4.5 (P = 0.020) and 9 (P = 0.018), but not different to samples at pH 5 (P = 0.773), 6 (P = 0.386), 7 (P = 0.773) and 8 (P = 0.564). There were significant differences in the concentration of fibre-bound CT at pH 4.5 compared with samples at pH values 5 (P = 0.020), 6 (P = 0.020), 7 (P =

0.020) 8 (P = 0.020) and 9 (P = 0.025). There were no significant differences in the concentration of fibre-bound CT at pH 5 compared with samples at pH values 6 (P = 0.773), 7 (P = 0.381) and 8 (P = 0.773). There were no significant differences in the concentration of fibre-bound CT at pH 6 compared with samples at pH values 7 (P = 0.773) and 8 (P = 0.773). There was a significant difference in the concentration of fibre-bound CT between pH 7 and 9 (P = 0.018) but not between samples at pH 7 and 8 (P = 0.564). There were significant differences in the concentration of fibre-bound CT between samples at pH 8 and 9 (P = 0.018).

The pH range at which CT is mostly in the free fraction is between pH 1-4, with the greatest amounts between pH 1.5-2.5 (Figure 7.1). The CT is forming a stable complex with protein at a pH range of 3-9 and also at pH 1 (Figure 7.1). The CT is a stable complex with fibre at pH 1, and also a small amount is complexed at pH 3-4 and also at 5-8 (Figures 7.1).

The amount of free, protein-bound and fibre-bound CT as a percentage of the total CT detected is shown in Table 7.8.

Table 7.8 Mean of concentration of free, protein-bound and fibre-bound condensed tannin (CT) at pH 1-9 expressed as a percentage of the total condensed tannin detected.

pH	Free CT (%)	Protein-bound CT (%)	Fibre-bound CT (%)
1	37	47	15
1.5	100	0	0
2	99	1	0
2.5	89	11	0
3	41	57	2
4	39	59	2
4.5	0	99	1
5	3	94	3
6	3	94	3
7	5	92	3
8	8	89	3
9	15	85	0

Protein formed a stable complex with CT at pH 1 (47% of total) and also between pH 3-9 (100-85% of total).The majority of the CT is unbound between pH values of 1.5-

2.5. Fibre-bound CT complexes only account for a very small percentage (< 3%) of total from pH 3-8 but was found to account for 15% of the total CT bound at pH 1.

7.4.3 Relationship between CT fractions and pH

The relationships between pH and free CT and pH and protein-bound CT can be seen in Figures 7.2 and 7.3.

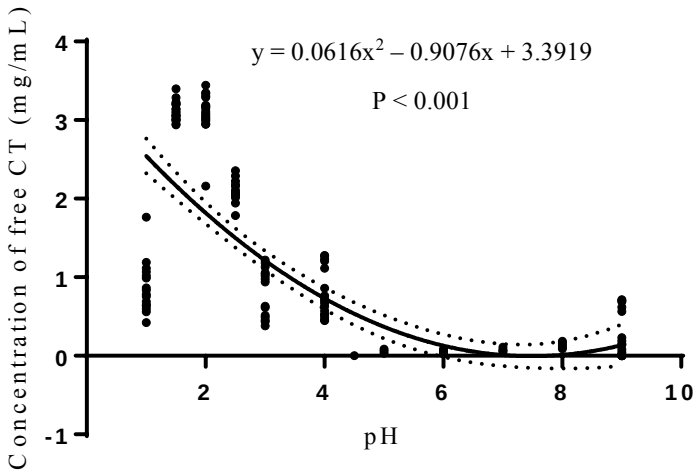


Figure 7.2 Relationship between pH and free condensed tannin (mg/mL) in *in vitro* samples at pH 1-9. Dashed line depicts 95% confidence intervals.

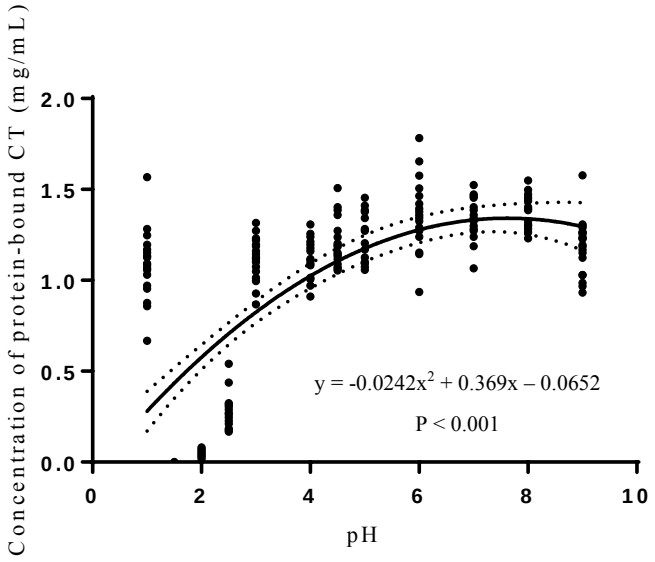


Figure 7.3 Relationship between pH and protein-bound condensed tannin (mg/mL) in *in vitro* samples at pH 1-9. Dashed line depicts 95% confidence intervals.

A highly significant, negative quadratic relationship was found to exist between pH and free CT ($P < 0.001$; r_s coefficient = -0.712; $r^2 = 0.569$) and a highly significant, positive quadratic relationship between pH and protein-bound CT ($P < 0.001$; r_s coefficient = 0.690; $r^2 = 0.484$). No significant relationship was found to exist between pH and fibre-bound CT ($P = 0.117$; r_s coefficient = 0.101; $r^2 = 0.083$).

7.4.4 Relationships between condensed tannin fractions

The relationship between CT fractions is shown in Figures 7.4 – 7.6.

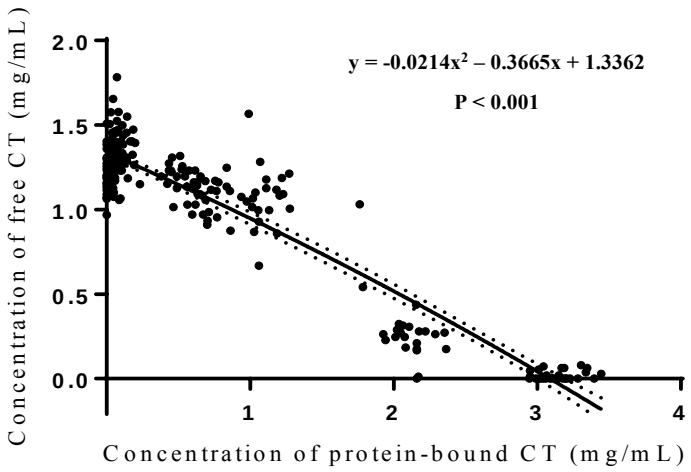


Figure 7.4 Relationship between free condensed tannin (CT) and protein-bound CT (mg/mL) in *in vitro* samples at pH 1-9. Dashed line depicts 95% confidence intervals.

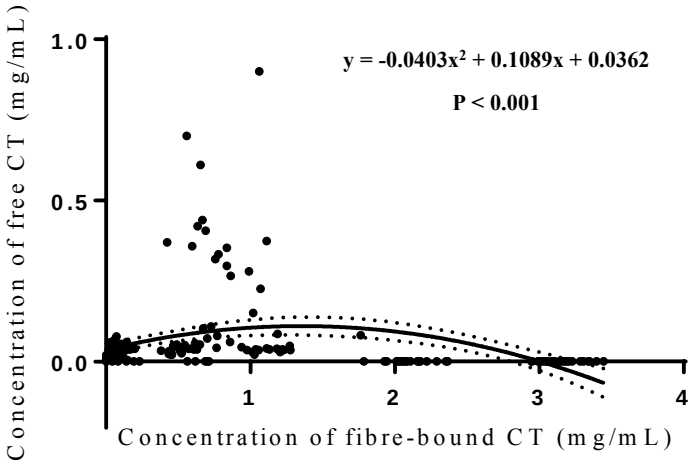


Figure 7.5 Relationship between free condensed tannin (CT) and fibre-bound CT (mg/mL) in *in vitro* samples at pH 1-9. Dashed line depicts 95% confidence intervals.

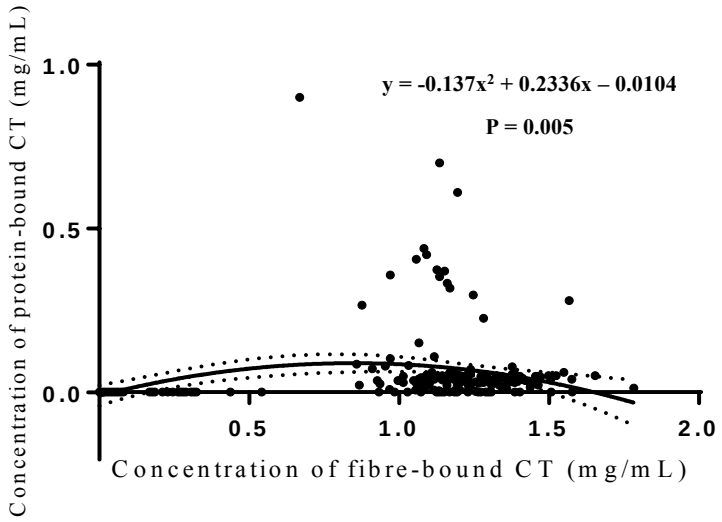


Figure 7.6 Relationship between protein-bound condensed tannin (CT) and fibre-bound CT (mg/mL) in *in vitro* samples at pH 1-9. Dashed line depicts 95% confidence intervals.

A highly significant, negative quadratic relationship was found to exist between free CT and protein-bound CT ($P < 0.001$; r_s coefficient = -0.732; $r^2 = 0.908$). The negative quadratic relationship, however significant ($P < 0.001$; r_s coefficient = -0.303; $r^2 = 0.125$), was only weak between free CT and fibre-bound CT. The positive quadratic relationship between protein-bound and fibre-bound CT was found to be very weak, however, still significant ($P = 0.005$; r_s coefficient = 0.183; $r^2 = 0.082$).

7.4.5 Total condensed tannin detected

Results for the calculated total CT concentration at pH 1-9 can be seen in Figure 7.7.

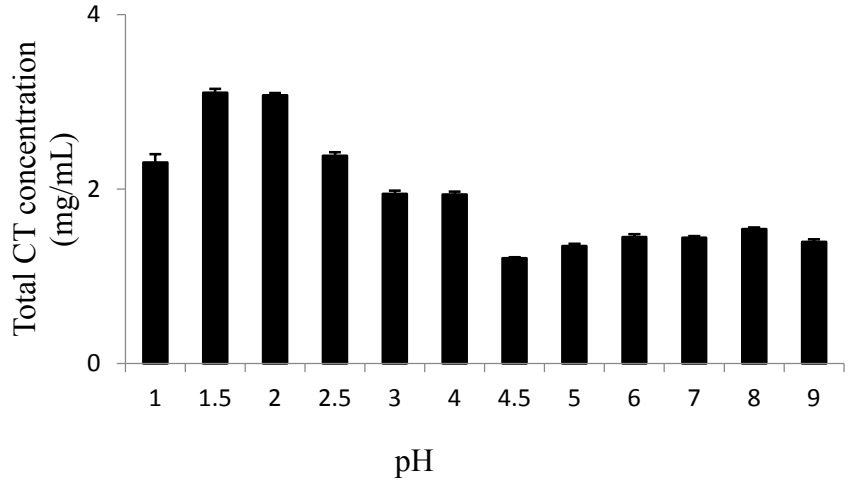


Figure 7.7 Means ± standard error of the means of the concentration (mg/mL) of total condensed tannin (CT) for pH 1-9.

The total CT concentration varied with pH, with the CT concentration tending to be greater between pH 1-2.5, which, results in a concentration greater than 2 mg/mL of CT, however, the values are significantly different at different pH values ($P < 0.001$).

7.4.6 Recovery of spiked condensed tannin

Results for total CT after spiking with 2 mg/mL CT only to each pH solution and water (Figure 7.8).

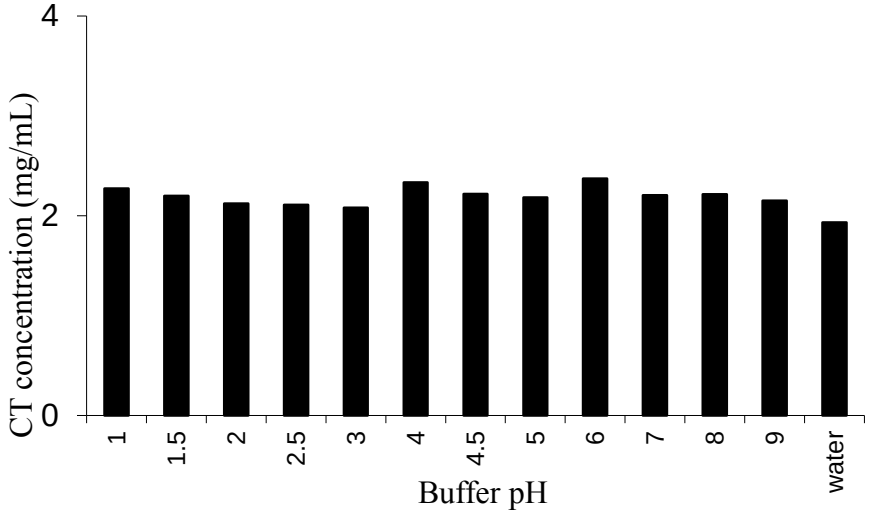


Figure 7.8. Concentration (mg/mL) of condensed tannin (CT) after spiking buffer at pH 1-9 and water with 2 mg/mL of CT.

The CT concentration between sample means was not significantly different ($P = 0.070$) with different buffer pH.

7.5 Discussion

Condensed tannin in plants can exist as protein-bound, fibre-bound and unbound (free). It is well known that these three fractions are interchangeable along the GI tract. Binding and dissociation of CT is influenced by pH, presence of plant and animal proteins, microbes and enzymes, as well as the presence of bile secretions in the duodenum.

7.5.1 Complexing time

The concentration of CT was determined after incubating samples for 0.5, 3, 8 or 24 h to determine if time exposed to CT would affect the amount of complexing with cellulose and Rubisco, *in vitro,* without the influence of digesta.

Overall complexing time did not have a significant effect (P = 0.996) on the concentration of free, protein-bound or fibre-bound CT in these *in vitro* assays (Tables 7.4-7.6). Hagerman and Robbins (1987) reported that 15 minutes complexing time was adequate for precipitation of purified CT compared to longer incubations for crude extracts. Contrary to these findings, other work has suggested that time exposed to CT has an effect on the amount of Rubisco complexed with CT (Jones and Mangan 1977, Terrill et al. 1994). Jones and Mangan (1977) found that there were significant increases in the amount of protein-bound CT after incubating CT extracted from sainfoin with Rubisco after 24 h compared to 2 h incubation at both 0 and 39ºC. They also reported that altering the ionic (0.05-0.50) and ascorbic acid strengths (0.10-1.0%) did not influence the amount of protein-bound CT complexes. In a study by Terrill et al. (1994), using sheep digesta spiked with CT, it was found that the samples incubated at 39ºC for 24 h compared to those incubated for 0 h had a greater amount of protein-bound CT complexes. This was the case for all digesta that was extracted from the rumen, abomasum, duodenum and ileum.

7.5.2 Influence of pH on complexing of condensed tannin

The concentration of the three fractions of CT (protein-bound, fibre-bound and free CT) were analysed *in vitro* after incubation at a range from pH values from 1-9 to determine the influence of pH on CT complexes with protein and fibre in the absence of any influencing constituents of GI digesta.

Complexing of CT depends on the molecular weight, tertiary structure, isoelectric point, and compatibility of binding sites of tannin and protein. Foo et al. (1996) and Foo et al. (1997), suggested that molecular weight has a significant effect on the reactivity between protein and CT, however, recent work by Naumann et al. (2013) has shown that CT molecular weight does not fully explain protein precipitation that varies amongst species of forage legumes studied. The interactions of the tannin protein complexes are also influenced by the reaction conditions such as temperature, pH, and tannin:protein ratios. These tannin complexes are reversible and can be disrupted by detergents or pH (Jones and Mangan 1977, Hagerman and Butler 1978). Proteins are usually precipitated by CT most efficiently at pH values near their isoelectric point. Rubisco is known to dissociate from CT at more alkaline and acid pH away from its isoelectric point (Douillard and Mathan 1994), which is approximately 5.5 (Jones and Lyttleton 1972) where the protein-protein electrostatic repulsion is minimal (Hagerman and Butler 1981).

pH did have a significant effect on the concentration of all three fractions of CT ($P < 0.001$). The CT extracted from calliandra was found to form a stable complex with the spinach Rubisco at pH 1 and from pH 3-9 (Figure 7.1). At pH 1, 47% of the total CT was complexed with protein and from pH 3-9 ranged from 57-99%, with 99% complexing occurring at pH 4.5, which is one pH unit below the isoelectric point at which the attraction would assume to be the greatest. The percent of protein complexing with CT as percent of total CT at pH 5 and 6 is still, however, high at 94%. *In vitro* studies by Perez-Maldonado et al. (1995) also found that protein-CT complexes were only formed at pH 3.5-5.5, the highest amount being formed at pH 4.5. This study also showed that the addition of rumen fluid shifted the pH to 6-6.5 at which protein was bound to CT. This is also in agreement with a study by McNabb et al. (1998) who investigated the effect of pH on precipitation of Rubisco without CT and found that at pH 5 about 40% was precipitated compared with only 10% at pH 5.5 (isoelectric point). This finding by McNabb et al. (1998), is interesting as Rubisco was dissolved in the buffer before the CT was added in this chapter and may have had minimal time to render some Rubisco unavailable due to precipitation. It was only minutes before the complex was added to the buffers and not a time lag of 90 min as shown by (McNabb et al. 1998). The results in this chapter did not appear

to have that same effect at pH 5 as most of the CT was in the protein-bound at this pH (Figure 7.1-7.4). Work by others have found a narrower range of stable complexes and many report that CT do not form stable complexes at alkaline pH greater than 7, although the Rubisco and CT are from different plants to that presented in this study (Jones and Mangan 1977, Mole and Waterman 1987, Perez-Maldonado et al. 1995, Diaz-Hernandez et al. 1997). Early *in vitro* work by Jones and Mangan (1977), involving complexing of CT extracted from sainfoin and with Rubisco from white clover showed that the most stable protein-CT complex occurred at pH 4-7. Also in agreement are results from the *in vitro* work by Diaz-Hernandez et al. (1997), who reported a stable complex was formed between CT from a range of shrubs and spinach Rubisco at a pH range of 3-7. In this study they reported that less complexing occurred after incubating the CT and Rubisco in abomasal and intestinal fluid, also confirmed with addition of commercial pepsin and trypsin, suggesting that proteolytic activity had a greater effect than pH alone on dissociation. McNabb et al. (1998) disagrees with the findings of others and reports a pH range from 3-8 at which the complex between protein from white clover and CT extracted from both *Lotus corniculatus* and *Lotus pedunculatus* is stable, which more closely agrees with the findings of this study. It has also been shown by Diaz-Hernandez et al. (1997) that after complexes formed between CT and Rubisco at pH 7, exposure to a range of buffers from pH 3-9 did not alter the amount that was originally complexed. This suggests that the initial conditions of complexing, usually in the rumen are important.

The fibre-bound CT as a percentage of the total CT was minimal with values recorded from 0-3% at pH 1.5-9. At pH 1 fibre-bound CT accounted for 15% of the total CT. At this pH the amount of CT in each of the three fractions was very different to what was present at pH 1.5 -2.5, the extreme acid conditions may be influencing the result.

The majority of the free CT was found to be in the pH range of 1.5-2.5, with 100% of the total CT detected in the unbound fraction at pH 1.5. There was a smaller percentage of free CT at pH 1 and also 3-4. Results of concentration of free CT at pH 1.5-2.5 demonstrate that more CT is detected in the assay than was actually added to the reaction, suggesting that at the lower pH range of 1.5-2.5 the assay is not very

reliable for free CT. The high amounts of CT in the free form corresponds to the low amounts in the protein-bound CT fraction and vice versa, confirming the interaction and interchangeability of these two forms of CT dependent on the pH of the assay.

7.5.3 Relationship between condensed tannin fractions and pH and within CT fractions

Significant relationships were found to exist between pH and free CT and between pH and protein-bound CT, however, no relationship existed between pH and fibre-bound CT. The free and protein-bound CT appear to be interchangeable as seen in Figure 7.4, however, only a small amount of CT is fibre bound except at pH 1, which may be due to other factors in such acidic conditions.

A highly significant, negative quadratic relationship was found to exist between free CT and protein-bound CT ($P < 0.001$; r_s coefficient = -0.732; $r^2 = 0.908$), confirming that these two fractions are interchangeable. Significant relationships were also found to exist between free CT and fibre-bound CT and between protein-bound and fibre-bound CT, however, these relationships were weak.

7.5.4 Total condensed tannin detected and pH

Some debate exists in the literature as to the effectiveness of the method used to assay CT. There has been problems reported in other studies with regards to losses and gains of CT along the GI tract and questions about the detectability of CT (Terrill et al. 1994, Perez-Maldonado and Norton 1996, Barrau et al. 2005). This suggests that conformational changes are rendering the CT undetectable and interfering factors in digesta and GI secretions are influencing the assay. The *n*-butanol-HCl method (Bate-Smith 1973, Porter et al. 1986) is specific for analysis of CT. The protein-bound, fibre-bound and free CT fractions of the tannin are able to be assayed using the modified method of Terrill et al. (1992b), based on Porter et al. (1986). It has been reported that this method can detect very low levels of CT but appears to be the most accurate at CT concentrations of 1% or greater. This method has also been modified by Perez-Maldonado (1994), for analysis of digesta and faecal samples, the author claiming the method of Terrill et al. (1992b) was not

sufficient in dissociating the CT from the protein, however, there still appears to be limitations to this method. The method used in this study is that of the modified method of Terrill et al. (1992b). As these were *in vitro* assays with no digesta there should be no interfering factors from digesta or GI secretions.

It has been suggested that the procyanidin content of the CT affects the assay, with a higher procyanidin content yielding a higher absorbance in the assay for the same tannin concentration (Kraus et al. 2003). This is in contrast to reports that there are no differences in the yield due to procyanidin and prodelphinidin composition of the extract (Porter et al. 1986). Foo et al. (1996) and Foo et al. (1997), however, suggested that the molecular weight of CT has a greater influence on reactivity with protein than the content of prodelphidin and procyanidin. If the CT contains a high proportion of delphinidin then the concentration of CT may be overestimated. Free CT seems to be the fraction that is being overestimated in the assay. Although only 2 mg of CT was added to the *in vitro* assay, the results for free CT at pH 1.5, 2 and 2.5 were all higher than 2 mg at 3.11, 3.05 and 2.11 mg, respectively. Total CT was also greater than 2 mg for pH 1 at 2.3 mg, with all fraction of CT contributing to this over estimation. Attempts were made to use a blank from each individual buffer but they were all zero, so the actual pH was not the problem, but the reaction. Each buffer as well as water alone in the absence of CT and cellulose was spiked with 2 mg of CT to see if pH had any effect on the reaction and so causing any over estimation of the results. The results showed variation around the concentration of 2 mg but this was most probably due to standard error. The difference between CT concentration at the pH range and also water were not significantly different. Further investigation into these assay anomalies are warranted.

7.6 Conclusion

It can be concluded that complexing times of 0.5, 3, 8 and 24 h had no significant effect on the amount of CT binding with protein *in vitro*. The pH range at which protein formed the most stable complex with CT was from 3-9. The most protein bound to CT as a percentage of total CT detected was 99% at pH 4.5. This study confirmed, that *in vitro* binding of CT with and dissociation from protein is

significantly pH dependant; however, this was not the case for the fibre-bound CT complexing.

CHAPTER 8 *IN VITRO* INHIBITION OF DIGESTIVE ENZYMES BY CALLIANDRA CONDENSED TANNIN.

8.1 Introduction

The shrub legume calliandra contains significant levels of CT and is also a potential source of high protein, with respect to ruminant nutrition in the tropics. Calliandra has a very good quality amino acid profile (Widiawati and Teleni 2004), however, a significant proportion of the calliandra protein is excreted as faeces (Perez-Maldonado and Norton 1996, Widiawati 2002, Cresswell 2007). Furthermore, DMD has been shown to decrease when feeding a diet containing CT (Perez-Maldonado and Norton 1996, Cresswell 2007, Aufrère et al. 2013).

Condensed tannins are capable of binding and/or precipitating water-soluble proteins (Bate-Smith 1973, Haslam 1989), and also forming complexes with minerals and carbohydrates, including cellulose. Condensed tannins thus have the potential to bind digestive enzymes, Rubisco or both (Mole and Waterman 1987), inhibiting protein release in the gut and therefore restricting protein utilization by the animal.

In vivo and *in vitro* studies from previous chapters have given insight into the dynamics of calliandra CT. *In vivo* studies (chapter 5) have confirmed that CT is binding to and dissociating from protein in different segments along the GI tract of lambs. This was also consistent with results from *in vitro* studies (chapter 7), in the absence of lamb digesta. Results from the *in vivo* work demonstrate that the greatest percent of protein-bound CT of all of the segments was found to exist in the rumen (51% of total CT detected). Rubisco is bound to CT and hence not available for microbial degradation in the rumen. This is desirable as the amino acid profile from microbial degradation, is not always as good as that from proteolytic degradation in the small intestine. There are nutritional advantages, however, only if this protected or bypass protein is subsequently made available for digestion by proteolytic enzymes in the abomasum and small intestine. The CT dynamics clearly demonstrated in the *in vivo* work that the majority of CT is in the free form in both the abomasum (95% of total CT detected) and the duodenum (55% of total CT detected). Samples were not able to be

collected from the jejunum but ileal samples indicated that 31% of total CT detected was in the protein-bound form. However, the protein was still not all available for digestion and absorption as most was found to be excreted in the faeces (Cresswell 2007). It was postulated then that the CT was somehow inhibiting the proteolytic enzymes or rendering the enzymes inactive. It is also postulated that some protein-bound CT was not being detected by the assay.

The experimental work in this chapter will determine if CT is also binding and inhibiting the activity of two key digestive enzymes, trypsin and pancreatic α-amylase. This may help explain why some protein is not available for digestion and absorption.

The aim of this experimental work was to determine if purified CT from calliandra binds to and causes inhibition of the digestive enzymes, trypsin and pancreatic α-amylase. Maximum activity and any inhibition of the trypsin and pancreatic α-amylase will be determined in the presence and absence of purified calliandra CT.

It was hypothesised that:

i. *Calliandra CT will inhibit the activity of the digestive enzymes, trypsin and pancreatic α-amylase.*

8.2 Materials and Methods

8.2.1 Location

This experiment was undertaken in the Animal Health and Production Nutrition laboratory at the College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville.

8.2.2 Condensed tannin

Purified CT extracted from calliandra was used in this study (chapter 3). A stock solution of 0.3 mg/mL was made by dissolving the lyophilized CT in 0.5 mL methanol and then made to volume with deionised water.

8.2.3 Trypsin assay

Trypsin inhibition was measured using a modified version of the method of Quesada et al. (1995), derived from the original method of Kakade et al. (1969). This colorimetric method measures the production of a yellow dye (p-nitroaniline) at 410 nm due to trypsin hydrolysis of the colourless synthetic dye, benzoyl-DL-arginine-p-nitroanilide (BAPNA). Assays were performed in triplicate and separate blanks for individual CT concentrations were used.
For the determination of maximum trypsin activity, 1 mL of a solution of 0.02 mg/mL trypsin (lyophilized trypsin from bovine pancreas, ≥ 10 000 BAEE units/mg protein, Sigma-Aldrich Pty Ltd, Sydney, Australia) in 0.001 N HCl was mixed with 1 mL of deionised water in a 5 mL tube. A 2.5 mL solution of BAPNA in Trizma buffer (pH 7.6 at 37º C) was added to the 5 mL tube, mixed and incubated for 10 min in a 37º C water bath (Memmert GmbH and Co, KG). The reaction was stopped by the addition of 0.5 mL of 30% acetic acid. Absorbance at 410 nm was read in a spectrophotometer (Genesis 10S UV-Vis spectrophotometer, Thermo fisher Scientific, Madison, WI, USA). This absorbance value was the maximum amount of yellow p-nitroaniline released (D). Blanks were prepared as above with the exception that acetic acid was added before the BAPNA.

For the determination of percent CT inhibition of trypsin the same procedure was performed as outlined above, but CT was used in the assay in place of the deionised water. The CT and trypsin were incubated for 10 min in a 37º C water bath, before the addition of the BAPNA to allow complexing. The amount of p-nitroaniline produced after inhibition was represented as (E). The concentration of CT used as inhibitor ranged from 0-0.3 mg/mL. The percentage inhibition was calculated using the following formula:

Percentage inhibition of trypsin = [(D-E) / D] x 100.

8.2.4 Pancreatic α-amylase Assay

Pancreatic α-amylase inhibition was measured using a modified method of Quesada et al. (1995) derived from the original method of Briggs (1967), with reductions in volume by half and also modified to add HCl to stop the enzymatic reaction (Xiao et al. 2006). Assays were performed in triplicate and blanks for each CT concentrations were used.

For the determination of maximum α-amylase activity, 0.5 mL solution of 0.00078 mg/mL α-amylase (α-amylase from porcine pancreas, type1-A, ≥ 1000 units/mg protein, Sigma-Aldrich Pty Ltd, Sydney, Australia) in 0.2% calcium acetate (pH 7.6 at 25ºC) was mixed with 0.5 mL of deionised water in a 5 mL tube. A 1.5 mL solution of starch (soluble starch from potato, Sigma-Aldrich Pty Ltd, Sydney, Australia) in phosphate buffer (pH 6.9 at 25ºC) was added to the 5 mL tube, mixed and incubated for 6 min in a 25ºC water bath (Memmert GmbH and Co. KG). The starch was solubilised by boiling in the phosphate buffer for 15 min and allowed to cool. The reaction was stopped by the addition of 1 mL of 1 M HCl. Fifty µL of the reaction mixture was pipetted into another 5 mL tube containing 5 mL of aqueous iodine solution (0.254 g of iodine and 4 g potassium iodide per litre of deionised water). Absorbance was read at 565 nm in a spectrophotometer (Genysis 10S UV-VIS spectrophotometer, Thermo Fisher Scientific, Madison, WI, USA). This absorbance value represents the unhydrolysed residual starch (C). To determine the amount of starch in the initial assay (A), the above protocol was followed with the enzyme being replaced with calcium acetate. The iodine solution was used as the blank.

For the determination of percentage inhibition of α-amylase by CT the same procedure was performed as outlined above, but CT was used in the assay in place of the deionised water. The CT and α-amylase were incubated for 30 min in a 25ºC water bath, before the addition of the starch solution to allow complexing. The amount of starch left after inhibition was represented as (B). The concentration of CT used as inhibitor ranged from 0-0.3 mg/mL. The percentage inhibition was calculated as follows:

Percentage inhibition of pancreatic α-amylase = [1-(A-B) / (A-C)] x 100.

8.3 Statistical Analysis

Data were tabulated and graphs generated using Microsoft Office Excel 2007 (Microsoft Corporation, USA). Spearman's correlations and quadratic regressions in SPSS were used to explore the relationship between percent inhibition and concentration of CT and the coefficient reported with 99.9% confidence intervals.

8.4 Results

8.4.1 Inhibition of digestive enzymes by calliandra condensed tannin

The effect of CT concentration on trypsin activity is presented in Figure 8.1. Trypsin activity was increasingly inhibited with increasing CT concentration. Maximum inhibition of 100% was achieved at a concentration of 0.20 mg/mL of CT and greater.

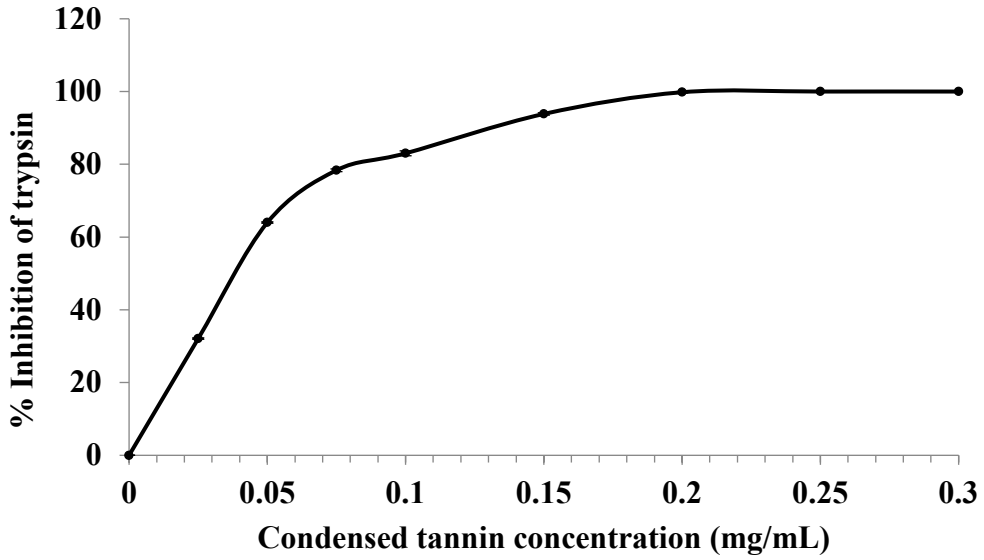


Figure 8.1 Trypsin inhibition curve for calliandra condensed tannin

The effect of CT concentration on pancreatic α-amylase activity is presented in Figure 8.2. Pancreatic α-amylase activity was increasingly inhibited with increasing CT concentration. Maximum inhibition of 96% was achieved at a concentration of 0.025 mg/mL of CT. Inhibition remained consistent at 96% for a CT concentration of 0.025 to 0.30 mg/mL.

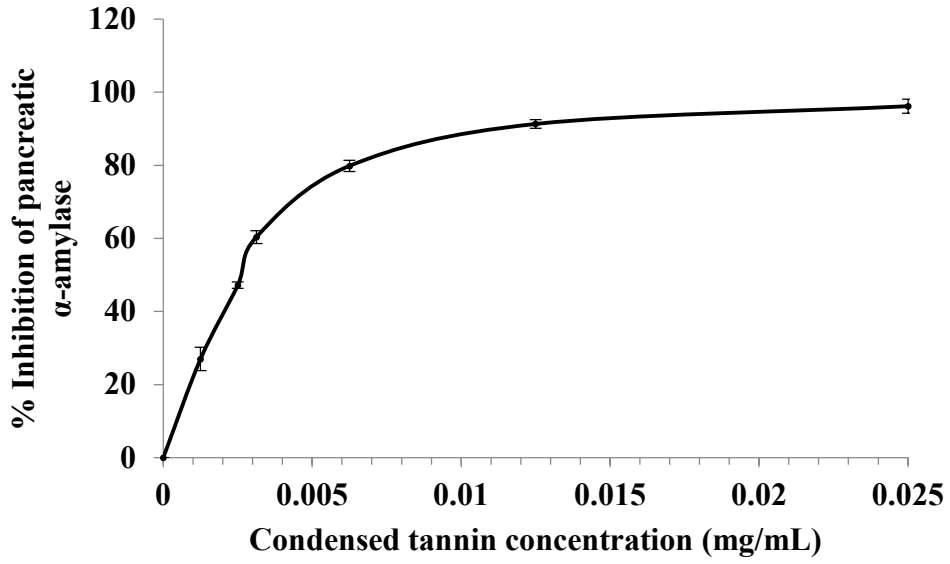


Figure 8.2 Pancreatic α-amylase inhibition curve for calliandra condensed tannin

8.4.2 Relationship between percent enzyme inhibition and concentration of CT

The relationship between inhibition of trypsin and concentration of CT can be seen in Figure 8.3. A highly significant, positive quadratic relationship was found to exist between percent inhibition of trypsin and concentration of CT ($P < 0.001$; r_s coefficient = 0.983; $r^2 = 0.965$).

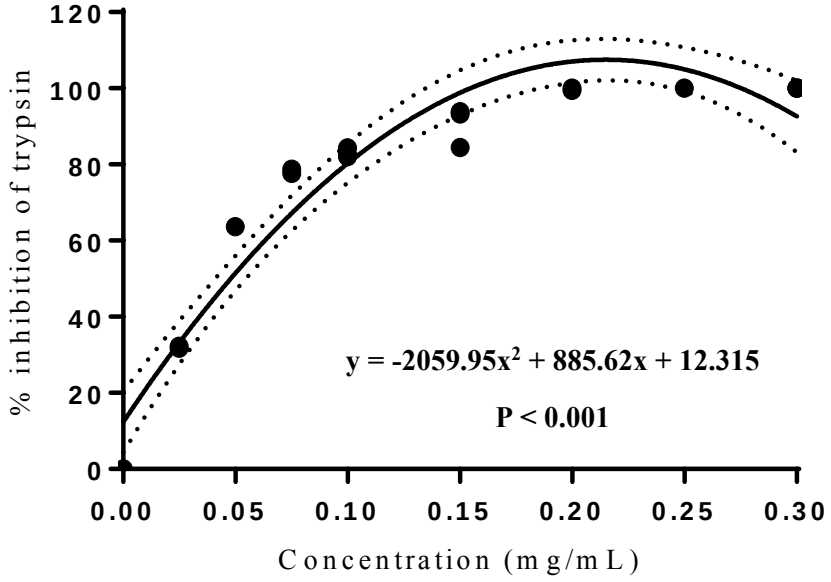


Figure 8.3 Relationship between percentage inhibition of trypsin and condensed tannin concentration (mg/mL). Dashed line depicts 95% confidence intervals.

The relationship between inhibition of pancreatic α–amylase and concentration of CT can be seen in Figure 8.4. A highly significant, positive quadratic relationship was found to exist between percent inhibition of pancreatic α–amylase and concentration of CT ($P < 0.001$; r_s coefficient = 0.984; $r^2 = 0.903$).

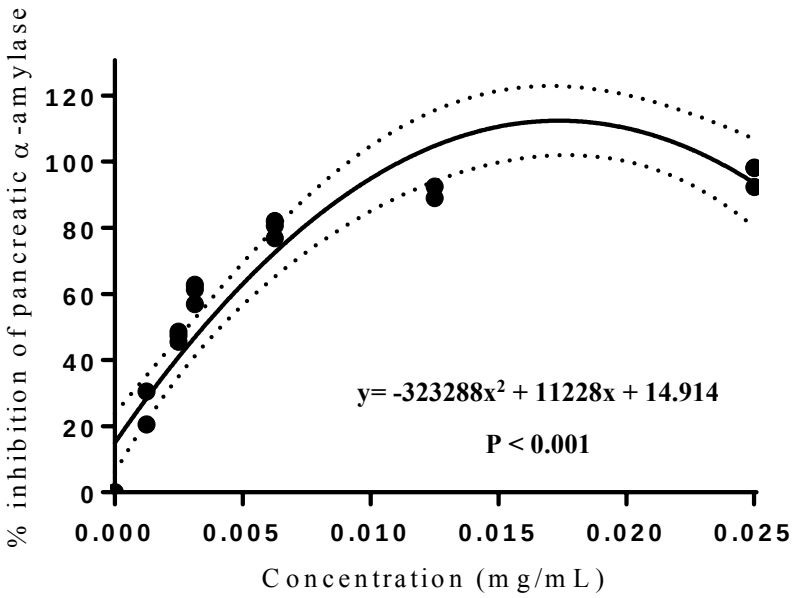


Figure 8.4 Relationship between percentage inhibition of pancreatic α-amylase and condensed tannin concentration (mg/mL). Dashed line depicts 95% confidence intervals.

8.5 Discussion

8.5.1 Enzyme inhibition

Condensed tannin and phenolic compounds have been reported to inhibit key abomasal, pancreatic and brush border digestive enzymes. Lentil, pear and cocoa extracts have been found to inhibit both trypsin and α-amylase (Quesada et al. 1995). Inhibition of lipase, α-amylase and phospholipase A_2 were reported to occur in the presence of cocoa, with the types of cocoa influencing the degree of inhibition (Gu et al. 2011). Trypsin and pepsin have been reported to dissociate some of the protein-CT complexes in abomasal and intestinal fluids (Diaz-Hernandez et al. 1997). Pancreatic α-amylase, maltase, sucrose, pancreatic lipase and pancreatic cholesterase esterase have been demonstrated *in vitro* to be inhibited by leaf extracts of *Moringa stenopetala* containing CT (Toma et al. 2014). Horigome et al. (1988) reported that a tannin diet compared to a non-tannin basal diet fed to rats did suppress the activity of trypsin and amylase, however, this was not the case for lipase, with activity in the

upper and lower GI segments unaffected. Results from this work also showed that the activity of lipase was actually enhanced in the middle GI segment.

The results obtained from this experimental work demonstrated that calliandra CT does inhibit the activity of the digestive enzymes, trypsin and pancreatic α-amylase. A highly significant, positive quadratic relationship was found to exist between percent inhibition of trypsin and concentration of CT (Figure 8.3). Condensed tannin was found to progressively inhibit trypsin until 100% inhibition was achieved at a concentration of 0.2 to 0.3 mg/mL CT (Figure 8.1). A highly significant, positive quadratic relationship was also found to exist between percent inhibition of pancreatic α–amylase and concentration of CT (Figure 8.5). Inhibition of pancreatic α–amylase occurred at a lower concentration of CT than the inhibition of trypsin. Inhibition of pancreatic α–amylase also increased progressively with CT concentration, however, only ever reached 96% inhibition at 0.025 mg/mL CT (Figure 8.2) to 0.3 mg/mL CT. Quesada et al. (1995) achieved approximately 95% inhibition using sorghum CT at a concentration of 0.06 mg/mL under similar assay conditions. Maximum inhibition of pancreatic α-amylase occurred at a 10 fold lower CT concentration than the maximum inhibition of trypsin, suggesting that CT had a much higher affinity for pancreatic α-amylase than trypsin.

8.5.2 Factors affecting enzyme activity and inhibition

The activity of an enzyme is related to several factors such as temperature, pH, substrate, enzyme, inhibitor and activator concentrations. These factors can either enhance or suppress the speed of the enzyme reaction. Enzyme inhibition will be driven by inhibitor and substrate concentrations and the relative affinities of the inhibitor and substrate for the active site of the enzyme.

The optimal pH is the pH range in which an enzyme is most active. The optimal pH for trypsin and pancreatic α-amylase are pH 7.8-8.7 and 6.7-7.0, respectively. The mean pH recorded in the ileum of lamb digesta (chapter 5) was 7.6, therefore it would be ideal to conduct the trypsin and pancreatic α-amylase assays near this pH. The trypsin assay was conducted at pH 7.6, which is slightly below optimum, however, consistent with that recorded for ileal digesta in chapter 5. The pancreatic α-amylase assay was conducted at pH

6.9 which is within the optimal pH range, however, much lower than pH 7.6 that was recorded for ileal pH in chapter 5. It was attempted to push the assay buffer up to pH 7.6, but the starch for the amylase assay would not stay in solution at this higher pH. Further studies using an alternative pancreatic α-amylase assay at pH 7.6 could be investigated.

There are also optimal temperature ranges at which enzyme reactions proceed. The rate of enzyme reactions is generally increased with increasing temperature. The temperature recorded for ileal pH at time of slaughter, mean of 4 lambs was 37.55ºC (Cresswell 2005), however, the trypsin assay and the pancreatic α-amylase assays were conducted at temperatures of 37 and 25ºC , respectively. This puts the trypsin assay in the optimal temperature range and the pancreatic α-amylase assay in what is termed the range of increasing activity. Most biological enzymes are rapidly denatured above 40ºC. The rate of inhibition was probably not altered by temperature, just the maximum pancreatic α-amylase reaction achievable.

Enzyme inhibition can be competitive, non-competitive, un-competitive or substrate inhibition. Competitive inhibition is when the inhibitor closely resembles the substrate and directly interacts with the active site of the enzyme. This inhibition is usually reversible with excess substrate displacing the inhibitor. Non-competitive inhibition occurs when the inhibitor exerts some effect on the enzyme, altering the active site so it cannot accept the substrate and can be either reversible or non-reversible. Un-competitive inhibition occurs when the inhibitor binds to the enzyme enhancing the binding of the substrate. Substrate inhibition occurs at a point when there are an overwhelming number of substrate molecules that block the enzymes active site. The concentration and activity of digestive enzymes, unfortunately, were not determined in the experiment conducted in chapter 5, so the CT: substrate:enzyme ratios *in vivo* are unknown. Enzyme kinetic studies to determine the nature of the calliandra CT inhibition and assays to determine digesta enzyme concentrations would be useful. The concentration of enzyme and CT inhibitor used were as per methods of trypsin and pancreatic α-amylase assays outlined above. This may not be a true representation as to what is occurring *in vivo*, (chapter 5). Condensed tannin concentration (% DM) in GI segments were measured in chapter 5 and GI segment volumes (L) collected by (Cresswell 2007) was used to estimate CT concentration in all GI segments of the lambs studied. Therefore, according to Cresswell (2007) the CT concentration in the ileum was

approximately 26 g/L, with a range from 9 g/L in the duodenum to 53 g/L in the abomasum. In the current study the CT concentrations in the inhibition assays were 0-0.3 mg/mL (0-0.3g/L), which is considerably lower (87 times less) than what was estimated *in vivo*. The trypsin assay was initially attempted using CT concentrations of 13, 26, 39 and 60 mg/mL, which resulted in 90, 79, 71 and 64 percent inhibition, respectively. This trend was opposite to what was expected as increasing the CT concentration resulted in decreasing inhibition. This assay was run three times to check for errors and confirm that this was the trend for inhibition.

These higher concentrations of CT may be causing conditions conducive to increased enzyme activity. Early work by McNabb et al. (1998) looking at tannin trypsin interactions in the proteolysis of BSA reported enhancement of trypsin activity at high tannin:protein ratios, suggesting that CT may be producing conformational changes to the substrate to make it more accessible to the trypsin. Ahmed et al. (1991) demonstrated a similar phenomenon with Cockerels that were fed a 0-25 g/kg Sal seed vegetable tannin diet (predominately HT), showed inhibition of α-amylase activity but when animals were fed a diet of 50 g/kg tannins, the amylase activity was no longer inhibited (Ahmed et al. 1991). Horigome et al. (1988) reported that a tannin diet compared to a basal diet fed to rats did suppress the activity of trypsin and amylase, however, this was not the case for lipase, with activity in the upper and lower GI segments unaffected. Results from this work also showed that the activity of lipase was actually enhanced in the middle GI segment (Horigome et al. 1988). These examples of inhibition causing enhancement may be attributed to the occurrence of un-competitive inhibition. Gu et al. (2011) reported inhibition of pancreatic lipase, pancreatic α–amylase and phospholipase A_2 by various types of cocoa. Kinetic studies from this work revealed that a mixed type of inhibition of pancreatic lipase was occurring with cocoa procyanidin pentamer, procyanidin deamer and regular cocoa. Non-competitive inhibition was found between phospholipase A_2 and cocoa procyanidin pentamer and procyanidin decamer. However, the inhibition type was found to be competitive between the regular coca and phospholipase A_2 (Gu et al. 2011). In a separate study by Petersen and Hill (1991), purified tannins from *Seracea Lespedeza* were found to non-competitively inhibit cellulose enzymes. Mixed non-competitive inhibition for α-amylase and α-glucosidase has been shown *in vitro* due to extracts from tannin containing *Azadirachta indica* (Kazeem et al. 2013). The inhibition of trypsin and pancreatic α–amylase by calliandra CT in this current study, for CT concentration

up to 0.3 mg/mL as shown in Figures 8.1 and 8.2 is most likely either competitive or non-competitive inhibition and at CT concentrations greater than 13 mg/mL might be explained by un-competitive inhibition, however, this type of inhibition is not common.

8.5.3 Consequences of CT interactions in the gut with digestive enzymes

It has been clearly demonstrated in this work that calliandra CT does inhibit the key digestive enzymes, trypsin and pancreatic α-amylase. This, in combination with evidence of binding of CT to Rubisco demonstrated *in vivo* (chapter 5, Table 5.12) and *in vitro* (chapter 7, Table 9.7) would account for the increased faecal N excretion and lowered DMD for animals fed a diet high in CT.

Pancreatic enzymes are secreted into the duodenum and then the majority of nutrients digested and absorbed in the jejunum and ileum. The amount of protein-bound CT in lamb digesta sampled in the *in vivo* study in chapter 5, changed from 6% in the duodenum to 31% in the ileum of total CT detected. Unfortunately, jejunum digesta was able to be collected but a pH of 6.3 was recorded. Extrapolating from the pH and CT data in chapter 5, a pH value of 6.3 would indicate that approximately 40-50% of total CT detected would be complexed with protein. This would indicate that the digestive enzymes would most likely be forming complexes with CT in the jejunum and ileum and not in the duodenum. In contrast to these results the *in vitro* studies from chapter 7 demonstrated that most of the CT remained protein-bound except at a very low pH of 1.5-2.5. The problem arising with the *in vitro* work in chapter 7 is assays were performed in the absence of lamb digesta, which would eliminate any interfering compounds that may alter complexing that are present in digesta. Bile acids have been reported to be excreted in greater amounts on diets containing kaki-tannin from Persimmons, both *in vitro* and *in vivo* using rats, suggesting that tannins are capable of binding bile acids and promoting their excretion (Matsumoto et al. 2011).Trypsin and pepsin have been reported to dissociate some of the protein-CT complexes in abomasal and intestinal fluids (Diaz-Hernandez et al. 1997) suggesting that not only pH has an effect on protein-CT dynamics but enzymes might also play a role in protein-CT complexing in the GI tract.

Dry matter digestibility was found to be significantly lower in the lambs fed a 100% calliandra diet at 43.3% compared to lambs fed a diet of lucerne pellets at 50.8% (Cresswell 2007). This was also the lambs that the digesta was sampled from in chapter 5. Work by Seresinhe and Iben (2003) using rumen simulation technique also found CT plants to have a lower DMD than lower CT containing plants. Their results for calliandra DMD was 39.5-53.5% compared to gliricidia containing only traces of CT with a DMD of 60-65%. The percent of diet fed, which equates to the CT concentration in the diet, appears to determine if DM digestibility is affected or not. There was no significant difference in DMD between a basal diet of 100% pangola grass compared to feeding sheep and goats calliandra at 300 g/kg DM with pangola grass (Perez-Maldonado and Norton 1996), suggesting there is a threshold of CT concentration above which DMD is affected. In another study sheep were fed diets containing 100% sainfoin (6.4% CT), 75% sainfoin and 25% lucerne (4.6% CT), 25% sainfoin and 75% lucerne (1.4% CT) and 0% sainfoin and 100% lucerne (0% CT), it was reported that the DM digestibility was 33.2, 46.9, 44.6 and 58%, respectively (Aufrère et al. 2013). It appears that diet from 75% to 25% sainfoin did not differ very much with respect to DMD, however, a large decrease was found with the 100% sainfoin diet. This also provides evidence that at a certain CT concentration DMD is lowered.

8.6 Conclusion

In can be concluded from the results of this current study, that calliandra CT does inhibit the digestive enzymes trypsin and pancreatic α-amylase, *in vitro*, with CT inhibiting pancreatic α-amylase at a much lower concentration compared to trypsin.

High levels of CT included in animal diets are capable of forming CT complexes in the small intestine with the digestive enzymes, trypsin and pancreatic α-amylase. In combination with evidence from previous work that CT also forms complexes with digesta protein *in vitro* (chapter 5) and Rubsico *in vivo* (chapter 7) may account for the lower DM digestibility and increased faecal N excretion reported by Cresswell (2007).

CHAPTER 9 *IN VITRO* ANTHELMINTIC EFFECTS OF PURIFIED CALLIANDRA CONDENSED TANNIN AND MIMOSA EXTRACTS ON *H. CONTORTUS* SUSCEPTIBLE AND MACROCYCLIC LACTONES RESISTANT STRAINS AND *T. COLUBRIFORMIS* SUSCEPTIBLE STRAIN.

9.1 Introduction

Gastrointestinal nematode infections are a serious threat to animal production with the effects of parasitism ranging from losses in productivity to death. *Haemonchus contortus* is the most pathogenic nematode of sheep and goats in tropical and subtropical regions with adult worms having the potential to extract 0.05 mL blood/worm/day (Rowe et al. 1988) eventually causing severe anaemia. *Trichostrongylus colubriformis* infection results in malnutrition, and causes black scours if present in large numbers.

Chemical anthelmintics used to control GI nematode infections are failing, with resistance to these anthelmintics alarming and now widespread (Love 2011). The use of fodder containing high levels of CT to control worm infections has shown promise. These nutraceuticals may be useful in both conventional and organic farming systems (Hoste et al. 2015). There are many reports of reduced egg production and establishment rates as well as a decrease in total worm numbers with feeding temperate forages (Niezen et al. 1995; Robertson et al. 1995), and also more recently tropical legumes (Cresswell 2007) containing CT. With worldwide increasing demand for chemical free meat and fibre, the use of CT as an anthelmintic is very attractive (Hoste and Torres-Acosta 2011, Hoste et al. 2015).

Tannins are naturally occurring water-soluble plant polyphenols (secondary plant metabolites), which have the ability to bind and precipitate proteins, minerals and carbohydrates, including cellulose. Condensed tannin can exist in three different forms in the plant as protein-bound, free and fibre-bound, with these fractions also being interchangeable and reversible along the GI tract. The CT-complexes binding and dissociation status is influenced by characteristics and chemistry of the tannin and the protein as well as pH, presence of plant and animal proteins, microbes, enzymes and the presence of bile secretions

in the duodenum (Jones and Mangan 1977, Hagerman and Butler 1981, Gu et al. 2011, Matsumoto et al. 2011, Niderkorn et al. 2012).

In vitro tannins have been shown to have effects on different stages of the nematode life cycle (Table A2.2). Variable results have been reported and are dependent on the type, purity and concentration of tannin from the plant extract and also differences due to nematode species tested. However, not all effects detected *in vitro* can be correlated with what happens *in vivo* due to the different physiological conditions present in the GI tract and the interactions with constituents of digesta and secretions.

Screening plant extracts for potential anthelmintic properties can effectively be done using a variety of *in vitro* assays such as egg hatch assays (EHA) (Le Jambre et al. 1976, Hunt and Taylor 1989, Coles et al. 1992), laval feeding inhibition assays (LFIA) (Geary et al. 1993, Alvarez-Sanchez et al. 2005), larval exsheathment assays (LEA) (Bauhard et al. 2006) and larval migration inhibition assays (LMIA) (Rabel et al. 1994, Gill et al. 1991, Barrau et al. 2005). These assays target different stages of the nematode life cycle, with most of these assays originally developed to test the efficacy of chemical anthelmintics.

This study compared the effect of purified calliandra CT (93.4% CT, as described in Chapter 3) and a commercial mimosa extract from *Acacia mearnsii* De Wild (black wattle), sold as a tanning powder containing 68.3-72.3 % tannins, on various stages of the life cycle of *H. contortus* and *T. colubriformis*. The black wattle naturally has a CT concentration of 6.15% DM (Carulla et al. 2005) compared to 12% DM in the calliandra used in this study.

In vitro EHA, LFIA, LMIA and LEA were conducted to demonstrate the direct effects that purified calliandra CT and mimosa extract from black wattle has on egg hatching, larval migration, larval exensheathment and also larval feeding. This was carried out in the presence of CT with negative controls of either water or PBS and positive controls of the anthelmintic agents Levamisole, Ivermectin or Thiabendazole. Eggs and infective third stage larvae (L3) were used in these assays from both a susceptible and macrocyclic lactones (MLs) resistant strain of *H. contortus* and a susceptible strain of *T. colubriformis*. CT concentrations of 200-1000 μg/mL CT were used as demonstrated by Cresswell (2007) to be suitable concentrations in dose response assays for egg hatch and larval development.

It is already confirmed that CT is binding to protein (Rubisco and digestive enzymes) in the GI tract of lambs (Chapters 5, 7 and 8), with the majority of the CT being bound to protein in the rumen and also post-duodenum. This work was conducted to determine if CT is affecting egg hatching, movement, exsheathment and feeding of *H. contortus* susceptible and MLs resistant strains and *T. colubriformis* susceptible strain, and if any variation occurs due to nematode species. Investigating the effect of CT in a variety of different in vitro assays gave insight into direct effects of CT on nematode eggs, first stage larvae (L1) and L3 and provide a better understanding of how CT can disrupt the lifecycle of these two nematode species.

The aim of this experimental work was to determine the *in vitro* anthelmintic effects of purified calliandra CT and mimosa extracts from black wattle.

It was hypothesised that:

i. *Calliandra CT and mimosa extract will decrease the rate of egg hatching, migration, exsheathment and feeding of H. contortus susceptible and MLs resistant strains and T. colubriformis susceptible strain.*

9.2 Materials and Methods

The current experiment was undertaken in the Nutritional Physiology and Metabolism unit and the Parasitology unit in the College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville.

9.2.1 Parasitology

Eggs and L3 from susceptable (Kirby strain) and GWBII MLs resistant strains of *H. contortus* and susceptable *T. colubriformis* (McMaster strain) were supplied by Veterinary Health Research Pty Ltd (Trevanna Road, West Armidale, NSW, 2350, Australia). The eggs purchased for the EHA had varying degrees of development, with the eggs of the *H. contortus* resistant and *T. colubriformis* susceptable strains containing advance larvae as seen in Plate 9.3 (A). The purity of the L3 used in the LEA were 100%, 99% and 87% for *H. contortus* susceptable, *H. contortus* MLs resistant and *T. colubriformis* susceptable strains,

respectively. The purity of the L3 use in the LMIA were 100%, 99% and 94% for *H. contortus* susceptable, *H. contortus* MLs resistant and *T. colubriformis* susceptable strains, respectively.

The *H. contortus* eggs and L3 were stored at 10°C and the *T. colubriformis* eggs and L3 were stored at 4°C until *in vitro* assays were conducted. Microscopic examination confirmed ensheathment of the L3 before assays were performed.

9.2.2 Experimental design

9.2.2.1 Tannin, eggs and larvae

Larvae and eggs were exposed to potential anthelmintic solutions of purified calliandra CT (93.4 % CT, as described in Chapter 3) and a mimosa extract from the bark of black wattle (68.3-72.3 % tannins, mimosa Africa Ltd, South Africa) at final concentrations in the tubes/wells of, 200, 400, 800 and 1000 µg/mL. All assays were performed in triplicate using 100 eggs or L3 per well or tube depending on the assay performed.

9.2.3 *In vitro* assays

9.2.3.1 Egg hatch assays

Egg hatch assays were performed in triplicate on eggs using the method described by Jackson and Hoste (2010), with the following modifications. The eggs and test solutions were placed directly into 5 mL plasma tubes that were sitting in the wells of 24 well plates. These were placed in the incubator in a rubber sealed plastic clip lock container with a saturated sponge in the base to give 100% relative humidity. Concentrated formaldehyde (1.5 mL)was added to each tube to preserve larvae until counting in place of helminthological iodine as in a trial period the iodine made the CT samples too dark and parasites difficult to count. For counting, the contents in the tubes were pipetted (3 mL) into 24 well plates that had previously been marked on the base with lines at 4 mm apart to enable counting in rows. The number of eggs and larvae (see Plate 9.3 B, C and D) were counted using an inverted microscope (Olympus-CK2, Olympus Corporation, Tokyo, Japan) and the % eggs hatched calculated using the following:

Eggs Hatched (%) = [(number of larvae completely out of egg shell) / (number of unhatched and partially hatched eggs + numbers of larvae completely out of egg shell)] x 100

Negative controls were deionized water and deionized water plus DMSO (10 µL of concentration ≥ 99.9%) and Thiabendazole (Sigma Cat. No. T8904, Sigma Aldrich, Castle Hill, NSW, Australia) as the positive control at a final concentration of 5 µg/mL.

9.2.3.2 Larval feeding inhibition assay

Larval feeding inhibition assays were performed in triplicate on eggs using the method described by Jackson and Hoste (2010), with the following modifications. Eggs were used in the assay and allowed to hatch to L1 to feed. Time from addition of eggs to stopping the assay was 44 h. Preliminary trials revealed that this was the optimum time for feeding to occur after hatching of L1. The eggs, test solution and Fluorescein isothiocyanate (FITC) were added at the same time into 5 mL plasma tubes and incubated on their sides in a clip lock plastic container at 25°C. The assay was stopped by the addition of 1 mL of concentrated formaldehyde instead of helminthological iodine, again due to the iodine making the samples too dark and parasites difficult to count. The samples were spun at 3000g (Eppendorf centrifuge 5702R, Eppendorf AG, Hamburg, Germany) in 15 mL graduated centrifuge tubes (Sarstedt AG and Co, Germany), and liquid aspirated off leaving approximately 60 µL in each tube. The 60 µL was pipetted onto a microscope slide, covered and numbers of fed and unfed larvae (see Plate 9.4 A and B) counted at100 x magnification using a stereo fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany). The percent inhibition of larval feeding calculated using the following:

Inhibition of Larval Feeding (%) = 100 - [(number of larvae fed) / (number of larvae fed + number of larvae unfed)] x 100

Negative controls were deionized water, deionized water plus DMSO (10 µL of concentration ≥ 99.9%) and positive controls were Thiabendazole (Sigma Cat. no. T8904, Sigma Aldrich, Castle Hill, NSW, Australia) and Ivermectin (Sigma Cat. no. 18898, Sigma Aldrich, Castle Hill, NSW, Australia), dissolved in DMSO and at a final concentration of 5 µg/mL.

9.2.3.3 Larval migration inhibition assay

Larval migration inhibition assays were performed in triplicate on ensheathed L3 using the method described by Jackson and Hoste (2010) with the following modifications. The ends of the pipette tips were cut off so as to provide a larger diameter to aspirate the L3. After filters were removed from wells each filter was carefully washed twice with 500 µL of deionized water and the remaining liquid containing the larvae pipetted out of the wells into 5 mL tubes. The larvae remaining on the filter that did not migrate was also washed twice with 500 µL of deionized water into a Petri dish. This liquid and larvae was pipetted into individually labelled tubes. Formaldehyde (40%, 1.5 mL) was added to each tube to preserve larvae until counting in place of helminthological iodine as in a trial period the iodine made the samples too dark and difficult to count in the presence of the calliandra CT and the mimosa extracts (see Plate 9.3 E and F). The contents in the tubes were transferred (3 mL disposable transfer pipette) into 24 well plates for counting that had lines marked on the base 4 mm apart to enable counting (see Plate 9.1). The larvae were counted using an inverted microscope at 40 x magnification and percent inhibition larval migration calculated using the following:

Larval Migration Inhibition (%) = 100 - [(number of larvae migrated through the nylon filter) / (number of larvae migrated through the nylon filter + number of larvae that did not migrate through the nylon filter)] x 100

At some of the higher CT and mimosa extract concentrations the samples were transferred into 4 cm Petri dishes with the bases marked with lines 4 mm apart so that the sample could be diluted to enable counting, as the CT and mimosa extract made visibility of the larvae difficult due to the brown colour and clumping of the tannins (Plate 9.3 (E) and (F)).

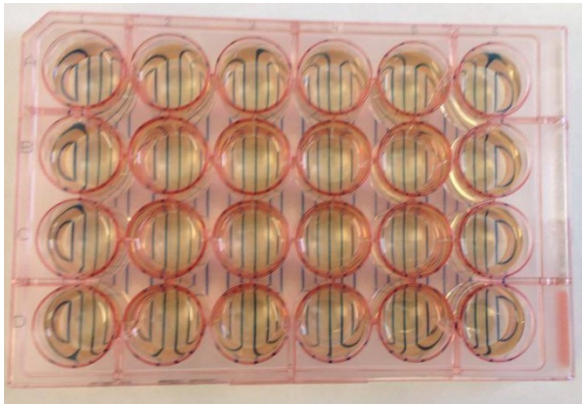


Plate 9.1 Twenty four well plates with bases marked at 4 mm intervals to enable counting.

Filter collars were made using modified 1.8 mL CryoPure tubes (Sarstedt AG and Co, Germany). A hole was drilled in the lid, 20 µm Nitex nylon mesh (Sefar Pty Ltd, Qld) was secured with the cryotube lid that had a hole drilled in it, and a rubber o-ring was placed on the outside of the tube to suspend the filter in the wells (see Plate 9.2 below).

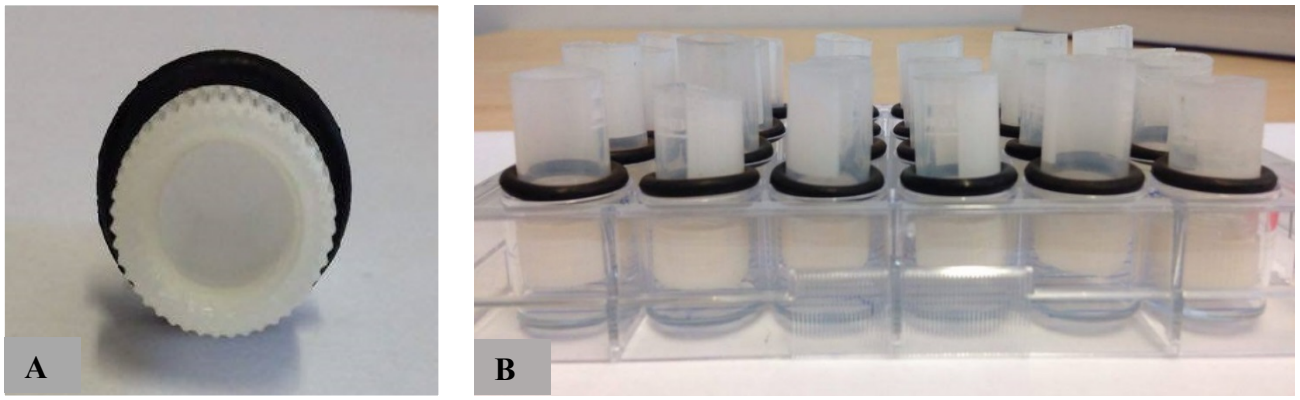


Plate 9.2 Nitex nylon mesh filter (A) and filters supended in 24 well plates with o-rings for support (B).

Negative and positive controls were deionized water and Levamisole Hydrochloride (Sigma # 31742, Sigma Aldrich, Castle Hill, NSW, Australia) at a final concentration in the wells of 5 µg/mL, respectively.

9.2.3.4 Larval exsheathment inhibition assay

Larval exsheathment inhibition assays were performed in triplicate on L3 using the method described by Jackson and Hoste (2010), with the following modifications. The ends of the pipette tips were cut off so as to provide a larger diameter to pipette up the L3. A 1:300 dilution of sodium hypochlorite (Sigma Cat. no. 239305, Sigma Aldrich, Castle Hill, NSW, Australia) equivalent to 0.013-0.017% available chlorine was predetermined as the optimum concentration to use as the exsheathment fluid to obtain gradual exsheathment over 60 min. Exposure times to the larval exsheathment fluid was 10, 20, 40 and 60 min. Assays were performed directly in 24 well plates that had previously been marked on the base with lines to enable counting, with PBS as the negative control. The end point was stopped with the addition of 4 µL of helminthological iodine. Samples were counted, ensheathed and exsheathed larvae (see Plate 9.5 A – C) using an Olympus inverted microscope (Olympus-CK2, Olympus Corporation, Tokyo, Japan) at 40 x magnification and percent larval exsheathment calculated using the following:

Larval Exsheathment (%) = [(number of larvae without sheath) / (number of larvae without sheath + number of larvae with a sheath or partially exsheathed)] x 100

9.3 Statistical Analysis

Raw data were initially tabulated in Microsoft Office Excel 2003 and treatment effects analysed in SPSS 22.0 for Windows (SPSS Inc., USA). Graphs were generated in GraphPad Prism 6 (GraphPad Software, Inc., USA). Separate analyses for calliandra CT and mimosa extracts were conducted because the calliandra CT was pure CT and the mimosa extract was an impure extract and of a lower tannin concentration.

Data for EHA for calliandra CT and mimosa extract were analysed using a General Linear Model in SPSS for effects of tannin concentration and worm strain on percent egg hatch, and where significance was found, data were then analysed using LSD test ($P \leq 0.05$). Data for the EHA for calliandra CT for effects of between tannin concentrations for each individual worm strain on percent egg hatch were analysed using a One-way ANOVA in SPSS, and where significance was found, data were then analysed using LSD test. Data for the comparison of negative and positive controls, and DMSO solvent for egg hatch were analysed using a General Linear Model in SPSS for effects of tannin concentration and worm strain, and where significance was found, data were analysed using LSD test.

Data for the LFIA for calliandra CT and mimosa extract were analysed using non-parametric Kruskall Wallace test for effects of tannin concentration and worm strain on inhibition of larval feeding and also for effects of CT for individual worm strains on inhibition of larval feeding. Where the Kruskell Wallace test found a significant effect ($P \leq 0.05$), the mean values were subjected to a Mann-Whitney test.

Pooled data LFIA for calliandra CT and Mimosa extracts were analysed using non-parametric Kruskall Wallace test for effects of tannin concentration on inhibition of larval feeding. Where the Kruskell Wallace test found a significant effect, the mean values were subjected to a Mann-Whitney test. Data for the comparison of negative and positive controls, and DMSO solvent for inhibition of larval feeding were analysed using non-parametric Kruskall Wallace test for effects of tannin concentration on inhibition of larval feeding. Where the Kruskell Wallace test found a significant effect, the mean values were subjected to a Mann-Whitney test.

Data for the LMIA for calliandra CT and mimosa extract were analysed using non-parametric Kruskall Wallace test for effects of tannin concentration and worm strain on inhibition of larval migration. Where the Kruskell Wallace test found a significant effect, the mean values were subjected to a Mann-Whitney test. Data for the LMIA for calliandra CT and mimosa extract were analysed using non-parametric Kruskall Wallace test for effects of negative and positive controls and DMSO solvent on inhibition of larval migration. Where the Kruskell Wallace test found a significant effect, the mean values were subjected to a Mann-Whitney test.

Data for the LEA for calliandra CT and mimosa extract were analysed using non-parametric Kruskall Wallace test for effects of tannin concentration, worm strain and time of exsheathment on inhibition of larval exsheathment. Where the Kruskell Wallace test found a significant effect, the mean values were subjected to a Mann-Whitney test. Data for LEA for calliandra CT and mimosa extracts were analysed using non-parametric Kruskall Wallace test for effects of tannin concentration and time of exsheathment for each worm stain on larval exsheathment. Where the Kruskell Wallace test found a significant effect, the mean values were subjected to a Mann-Whitney test.

Pearson's correlations and quadratic regressions in SPSS were used to explore the relationship between percent egg hatch and concentration of tannins for worm strains. Spearman's correlations and quadratic regressions in SPSS were used to explore the relationship between percent larval migration inhibition and concentration of tannins for worm strains. Effects were considered significant at a probability value of $P < 0.05$.

9.4 Results

9.4.1 Egg hatch assays using calliandra condensed tannin

Data for percent egg hatch of eggs from *H. contortus* susceptible, *H. contortus* MLs resistant and the *T. colubriformis* susceptible worm strains after incubation with calliandra CT are presented in Table 9.1 and Figure 9.1 below.

Table 9.1 Means ± standard error of the means for egg hatch (%) of eggs from *H. contortus* susceptible, *H. contortus* MLs resistant and the *T. colubriformis* susceptible strains after incubation with calliandra condensed tannin (CT), Thiabendazole and DMSO.

Calliandra CT (µg/mL)	***H. contortus* (susceptible)**	***H. contortus* (resistant)**	***T. colubriformis* (susceptible)**
0	80.51a ± 2.94	80.33a ± 2.01	93.54a ± 0.74
200	69.52a,c ± 4.38	97.50b,c ± 0.78	94.31a ± 0.23
400	64.16b,c ± 2.81	95.77b,d ± 3.33	87.94a ± 2.15
800	61.60b ± 3.84	90.05a,c ± 1.08	77.57b ± 3.09
1000	52.77b ± 3.93	70.57b ± 3.51	70.45b ± 3.98
P value	0.004	0.000	0.000
0	80.51a ± 2.94	80.33a ± 2.01	93.54a ± 0.74
0 + Thiabendazole	3.03b ± 3.03	21.98b ± 0.48	16.31b ± 2.55
0 + DMSO	82.45a ± 3.32	72.64a ± 2.61	93.32a ± 0.96
P value	< 0.001	< 0.001	< 0.001

Rows above the dotted line within each column with different letters are significantly different ($P < 0.05$).
Rows below the dotted line within each column with different letters are significantly different ($P < 0.05$).

The percentage of eggs that hatched after incubation in calliandra CT was significantly affected by both worm strain ($P < 0.001$) and concentration of CT ($P < 0.001$). There was a significant difference in percent egg hatch between the *H. contortus* susceptible strain and the *H. contortus* MLs resistant strain ($P < 0.001$) and also between the *H. contortus* susceptible strain and the *T. colubriformis* susceptible strain ($P < 0.001$). There was no significant difference in percent egg hatch between the *H. contortus* MLs resistant strain and the *T. colubriformis* susceptible strain ($P = 0.421$).

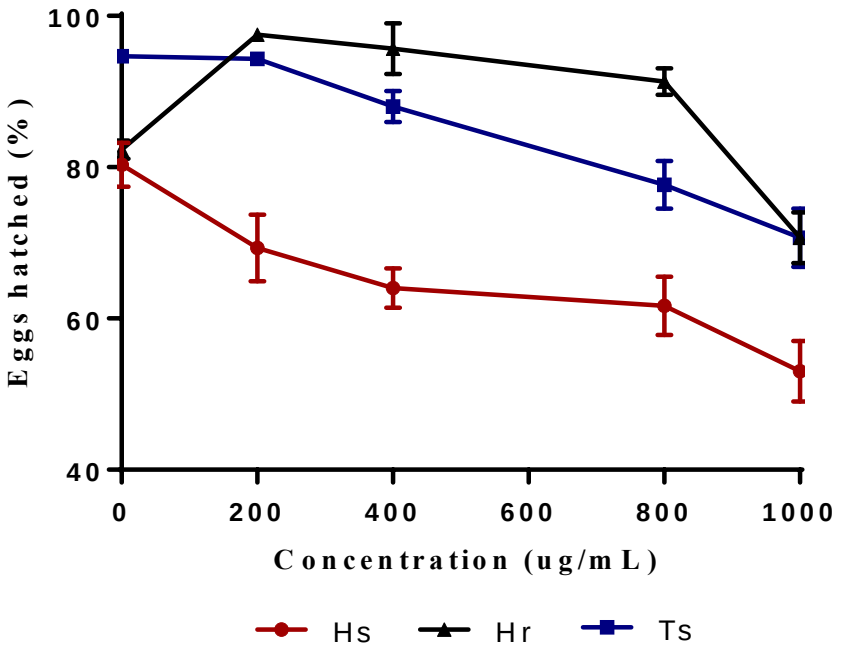


Figure 9.1 Means ± standard error of the means for egg hatch (%) of eggs from *H.contortus* susceptible (Hs), *H. contortus* MLs resistant (Hr) and the *T. colubriformis* susceptible (Ts) strains after incubation with calliandra condensed tannin.

The percent egg hatch for the negative control (no added CT) for the *H. contortus* susceptible, *H. contortus* and the *T. colubriformis* susceptible strains was 81%, 80% and 94%, respectively. For the *H. contortus* susceptible strain there was a significant decrease in the percent egg hatch between the negative control and CT concentrations of 400 ($P = 0.007$), 800 ($P = 0.003$) and 1000 µg/mL ($P < 0.001$), respectively, but not between the negative control and the 200 µg/mL ($P = 0.220$). For the *H. contortus* MLs resistant strain there was a significant decrease in percent egg hatch between the negative control and the 1000 µg/mL CT concentration ($P = 0.007$), no difference between the negative control and the 800 µg/mL CT ($P = 0.050$), but a significant increase in percent egg hatch between the negative control and CT concentrations of 200 and 400 µg/mL ($P = 0.003$). For the *T. colubriformis* susceptible strain there was a significant difference in the percent egg hatch between the negative control and CT concentrations of 800 ($P < 0.001$) and 1000 µg/mL ($P < 0.001$), respectively, but not between the negative control and the 200 ($P = 0.924$) or 400 µg/mL ($P = 0.101$).

9.6.1.1 Relationship between percent egg hatch and calliandra condensed tannin

Incubation of eggs in calliandra CT caused a dose-dependent egg hatch effect on the *H. contortus* susceptible strain and the *T. colubriformis* susceptible strain, with a decrease in percent egg hatch as CT concentration increased. The relationship between percent egg hatch and calliandra CT concentration for *H. contortus* susceptible and *T. colubriformis* susceptible strains can be seen in Figures 9.2 and 9.3, respectively.

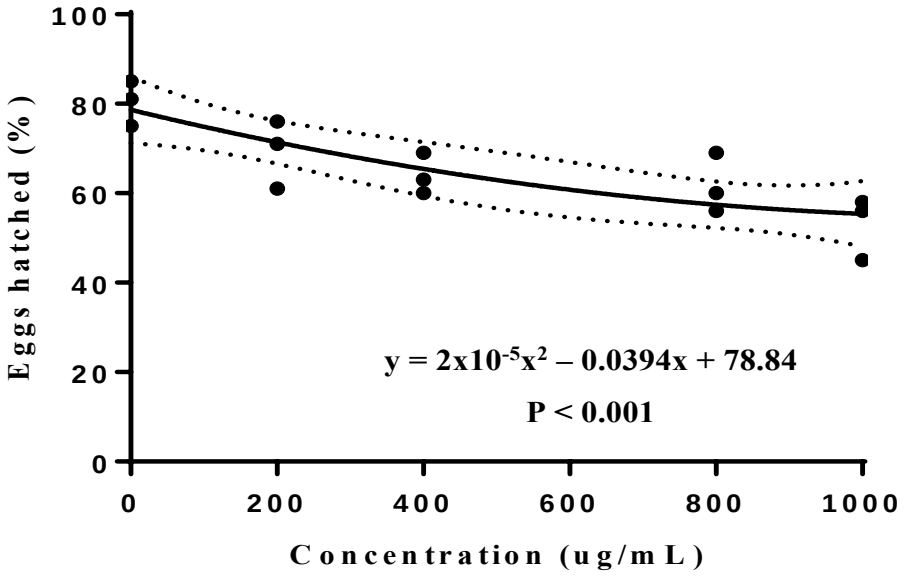


Figure 9.2 Relationship between percent egg hatch and calliandra condensed tannin concentration (µg/mL) for *H. contortus* susceptible strain. Dashed line depicts 95% confidence intervals.

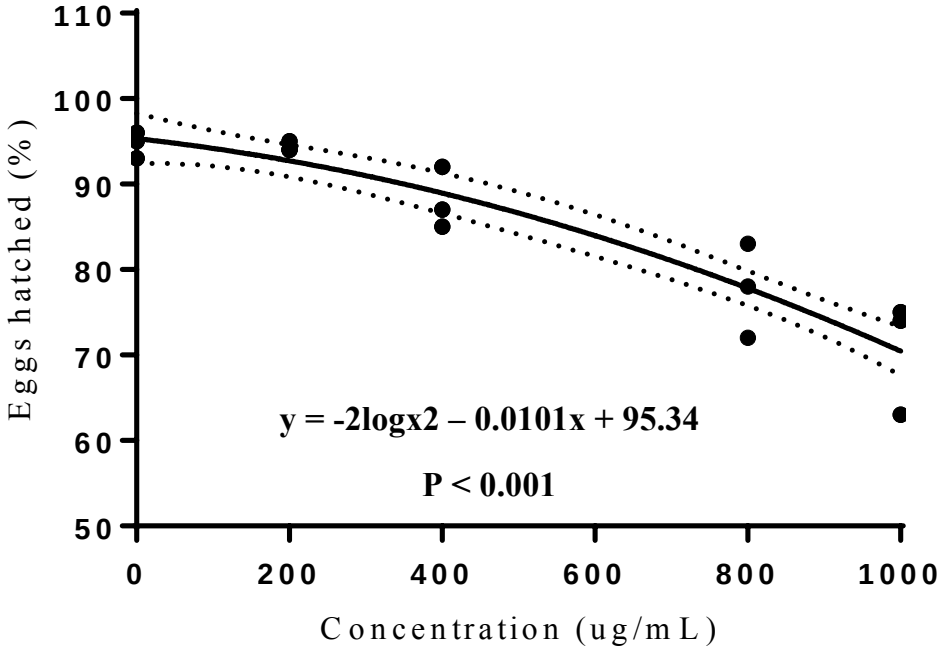


Figure 9.3 Relationship between percent egg hatch and calliandra condensed tannin concentration (µg/mL) for the *T. colubriformis* susceptible strain. Dashed line depicts 95% confidence intervals.

A highly significant, negative quadratic relationship was found to exist between percent egg hatch and CT concentration for both the *H. contortus* susceptible strain ($P < 0.001$; r coefficient = - 0.853; $r^2 = 0.702$) and the *T. colubriformis* susceptible strain ($P < 0.001$; r coefficient = - 0.907; $r^2 = 0.874$). No significant relationship was found to exist between percent egg hatch and CT concentration for the *H. contortus* MLs resistant strain ($P = 0.164$).

9.4.2 Egg hatch assays using mimosa extract

Data for percent egg hatch from the *H.contortus* susceptible, the *H. contortus* MLs resistant and the *T. colubriformis* susceptible worm strains after incubation with mimosa extract are presented in Table 9.2 and Figure 9.4 below.

Table 9.2 Means ± standard error of the means for egg hatch (%) of eggs from *H. contortus* susceptible, *H. contortus* MLs resistant and the *T. colubriformis* susceptible strains after incubation with mimosa extract, Thiabendazole and DMSO.

Mimosa Extract (µg/mL)	***H. contortus* (susceptible)**	***H. contortus* (resistant)**	***T. colubriformis* (susceptible)**
0	80.51a ± 2.94	80.33a ± 2.01	93.54a ± 0.74
200	68.67b,c ± 0.18	84.07a,c ± 3.50	91.05a ± 2.46
400	62.78b,c ± 2.11	92.90b,c,d ± 1.68	94.26a ± 1.75
800	59.45b,c ± 2.86	85.80a,d ± 2.86	94.47a ± 0.94
1000	50.56b ± 6.37	86.9a9 ± 2.46	93.50a ± 0.30
P value	0.002	0.051	0.237
0	80.51a ± 2.94	80.33a ± 2.01	93.54a ± 0.74
0 + Thiabendazole	3.03b ± 3.03	21.98b ± 0.48	16.31b ± 2.55
0 + DMSO	82.45a ± 3.32	72.64a ± 2.61	93.32a ± 0.96
P value	< 0.001	< 0.001	< 0.001

Rows above the dotted line within each column with different letters are significantly different ($P < 0.05$).
Rows below the dotted line within each column with different letters are significantly different ($P < 0.05$).

The percentage of eggs that hatched after incubation in mimosa extract was significantly affected by both worm strain ($P < 0.001$) and concentration of CT ($P = 0.001$). There was a significant difference in percent egg hatch between the *H. contortus* susceptible strain and the *H. contortus* MLs resistant strain ($P < 0.001$) and also between the *H. contortus* susceptible strain and the *T. colubriformis* susceptible strain ($P < 0.001$). There was no significant difference in percent egg hatch between the *H. contortus* MLs resistant strain and the *T. colubriformis* susceptible strain ($P = 0.302$).

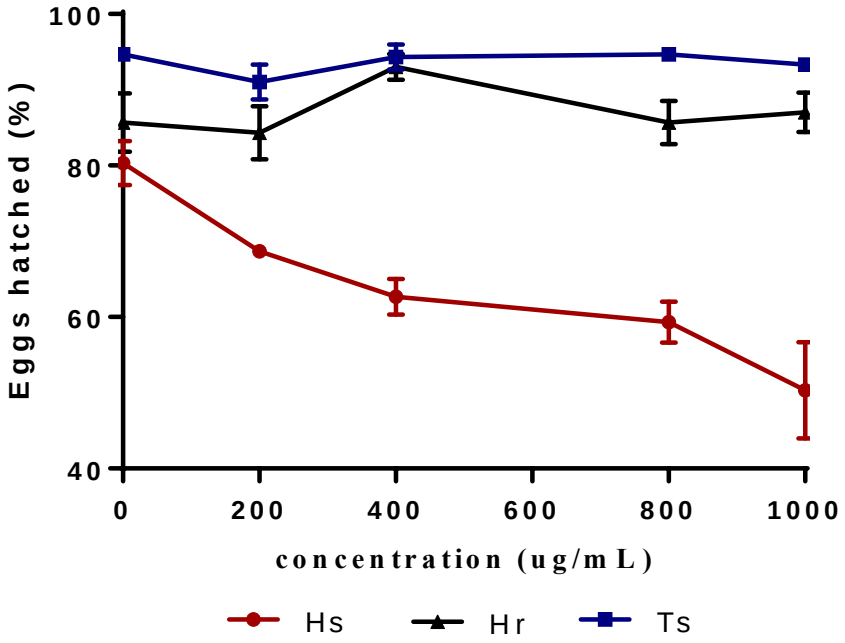


Figure 9.4 Means ± standard error of the means for egg hatch (%) of eggs from *H.contortus* susceptible (Hs), *H. contortus* MLs resistant (Hr) and the *T. colubriformis* susceptible (Ts) strains after incubation with mimosa extract.

The *H. contortus* susceptible strain was the only strain that showed a significant decrease in the percent egg hatch when incubated with mimosa extract ($P = 0.002$). There was no significant difference in percent egg hatch after incubation with mimosa extract for the *H. contortus* MLs resistant ($P = 0.051$) and the *T. colubriformis* susceptible ($P = 0.237$) strains.

The percent egg hatch for the negative control (no added CT) for the *H. contortus* susceptible strain was 81%. For the *H. contortus* susceptible strain there was a significant decrease in the percent egg hatch of the negative control and mimosa extract concentrations of 200 ($P = 0.039$), 400 ($P = 0.005$), 800 ($P = 0.002$) and 1000 µg/mL ($P < 0.001$), respectively.

9.4.2.1 Relationship between percent egg hatch and mimosa extract

Incubation of the eggs in mimosa extract caused a dose-dependent egg hatch effect on the *H. contortus* susceptible strain only, with a decrease in percent egg hatch with an increase in mimosa extract concentration. The relationship between percent egg hatch and mimosa extract concentration for *H. contortus* susceptible strain can be seen in Figure 9.5.

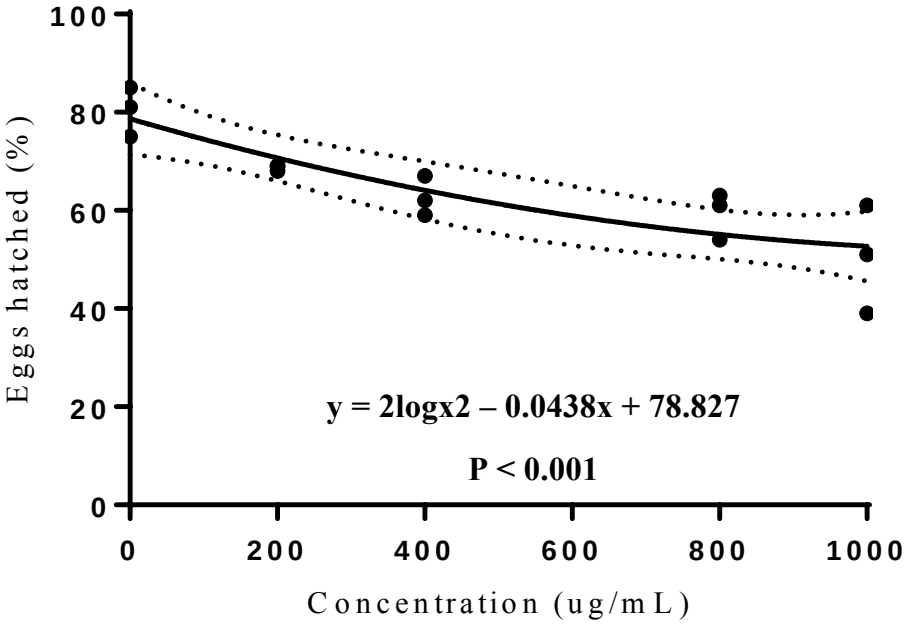


Figure 9.5 Relationship between percent egg hatch and mimosa extract concentration (µg/mL) for *H .contortus* susceptible strain. Dashed line depicts 95% confidence intervals.

A highly significant, negative quadratic relationship was found to exist between percent egg hatch and mimosa extract concentration for the *H. contortus* susceptible strain ($P < 0.001$; r coefficient = - 0.878; $r^2 = 0.748$). No significant relationship was found to exist between percent egg hatch and mimosa extract concentration for the *H. contortus* MLs resistant strain ($P = 0.752$) or the *T. colubriformis* susceptible strain ($P = 0.324$).

The inclusion of DMSO as the solvent for the anthelmintic Thiabendazole was not different to the results for the negative controls of zero concentrations of calliandra CT and mimosa extract for egg hatch for *H. contortus* susceptible strain ($P = 0.674$), *H. contortus* MLs resistant strain ($P = 0.189$) and the *T. colubriformis* susceptible strain ($P = 0.577$). However, the inclusion of the anthelmintic Thiabendazole as the positive control had a significant effect on percent egg hatch compared to the negative controls for all worm strains ($P < 0.001$).

9.4.3 Larval feeding inhibition assays using calliandra condensed tannin

Data for feeding inhibition of L1 of *H. contortus* susceptible, *H. contortus* MLs resistant and the *T. colubriformis* susceptible worm strains after incubation with calliandra CT are presented in Table 9.3 and Figure 9.6 below.

Table 9.3 Means ± standard error of the means for inhibition of larval feeding (%) for L1 of *H. contortus* susceptible, *H. contortus* MLs resistant and the *T. colubriformis* susceptible strains after incubation with calliandra condensed tannin (CT), Thiabendazole, Ivermectin and DMSO.

Calliandra CT (µg/mL)	***H. contortus* (susceptible)**	***H. contortus* (resistant)**	***T. colubriformis* (susceptible)**
0	31.11 ± 26.27	50.33 ± 17.95	20.00 ± 13.55
200	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
400	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
800	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
1000	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
P value	0.008	0.008	0.008
0	31.11 ± 26.27	50.33 ± 17.95	20.00 ± 13.55
0 + Thiabendazole	66.67 ± 33.33	66.67 ± 33.33	26.79 ± 8.81
0 + Ivermectin	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0 + DMSO	75.30 ± 9.87	75.30 ± 9.87	55.95 ± 22.62

The percentage inhibition of larval feeding after exposure to calliandra CT was significantly affected by concentration of CT (P = 0.001) but not by worm strain (P = 0.984). The feeding of the L1 was inhibited by 100% for all calliandra CT concentrations from 200-1000 µg/mL for *H. contortus* susceptible, *H. contortus* MLs resistant and the *T. colubriformis* susceptible worm strains.

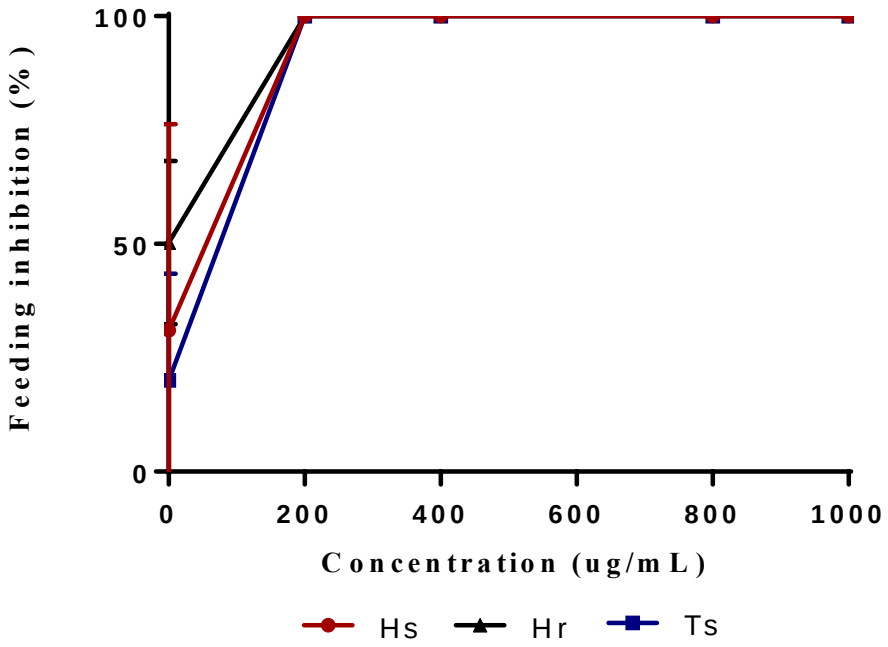


Figure 9.6 Means ± standard error of the means for feeding inhibition (%) of L1 from *H. contortus* susceptible (Hs), *H. contortus* MLs resistant (Hr) and the *T. colubriformis* susceptible (Ts) strains after incubation with calliandra condensed tannin.

Data for feeding inhibition after incubation with calliandra CT and pooled for worm strain are presented in Figure 9.7 and Table 9.4 below.

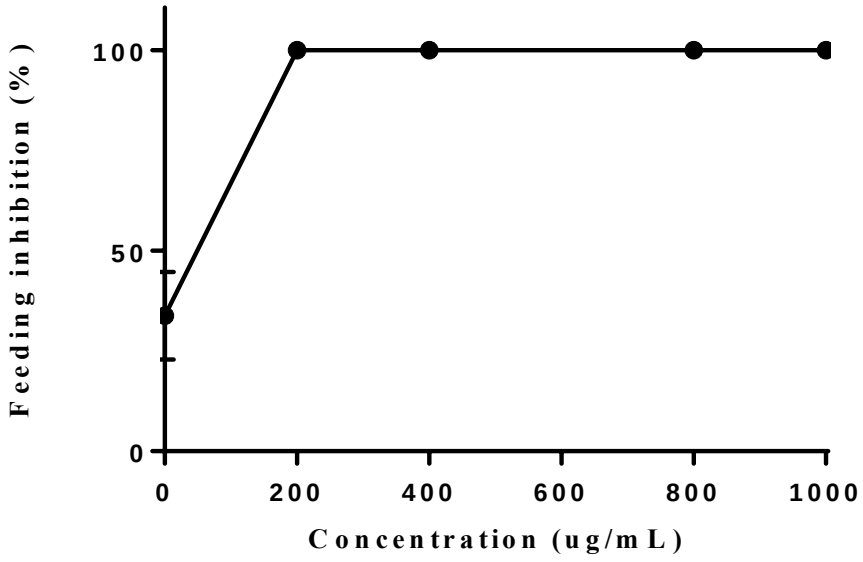


Figure 9.7 Means ± standard error of the means for inhibition of larval feeding (%) for L1 after incubation with calliandra condensed tannin. Data are pooled for worm strain.

Table 9.4 Means ± standard error of the means for inhibition of larval feeding (%) for L1after incubation with calliandra condensed tannin (CT), Thiabendazole, Ivermectin and DMSO. Data are pooled for worm strain.

Calliandra CT (µg/mL)	**Feeding inhibition (%)**
0	33.82a ± 10.92
200	100.00b ± 0.00
400	100.00b ± 0.00
800	100.00b ± 0.00
1000	100.00b ± 0.00
P value	< 0.001
0	33.82a ± 10.92
0 + Thiabendazole	53.38a,d ± 25.16
0 + Ivermectin	100.00b,c ± 0.00
0 + DMSO	68.85b,d ± 42.36
P value	< 0.001

Rows above the dotted line within each column with different letters are significantly different ($P < 0.05$).
Rows below the dotted line within each column with different letters are significantly different ($P < 0.05$).

The percent inhibition of larval feeding for the negative control (no added CT) pooled for worm strain was 34%. For the pooled data for worm strain, there was a significant increase in the percent inhibition of larval feeding between the negative control and CT concentrations of 200-1000 µg/mL ($P < 0.001$). There were no significant differences ($P = 1.000$) in inhibition of larval feeding between CT concentrations of 200, 400, 800 and 1000 µg/mL.
The inclusion of DMSO as the solvent for the anthelmintic Thiabendazole was different ($P = 0.038$) to the results for the negative controls of zero concentrations of calliandra CT for inhibition of larval feeding. The inclusion of the anthelmintic Ivermectin as the positive control had a significant effect on larval feeding compared to the negative controls ($P < 0.001$). Inhibition of feeding by Ivermectin was 100%. Thiabendazole was also used as another positive control, however, the inhibition of feeding was not different to the negative controls ($P = 0.166$).

9.4.4 Larval feeding inhibition assays using mimosa extract

Data for feeding inhibition of L1 of *H. contortus* susceptible, *H. contortus* MLs resistant and the *T. colubriformis* susceptible worm strains after incubation with mimosa extract are presented in Table 9.5 and Figure 9.8 below.

Table 9.5 Means ± standard error of the means for inhibition of larval feeding (%) of L1 of *H. contortus* susceptible, *H. contortus* MLs resistant and the *T. colubriformis* susceptible strains after incubation with mimosa extract, Thiabendazole, Ivermectin and DMSO.

Mimosa Extract (µg/mL)	***H. contortus* (susceptible)**	***H. contortus* (resistant)**	***T. colubriformis* (susceptible)**
0	32.86 ± 8.92	60.79 ± 11.80	64.97 ± 8.72
200	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
400	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
800	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
1000	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
P value	0.008	0.008	0.012
0	31.11 ± 26.27	50.33 ± 17.95	20.00 ± 13.55
0 + Thiabendazole	66.67 ± 33.33	66.67 ± 33.33	26.79 ± 8.81
0 + Ivermectin	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0 + DMSO	75.30 ± 9.87	75.30 ± 9.87	55.95 ± 22.62

Rows above the dotted line within each column with different letters are significantly different ($P < 0.05$).
Rows below the dotted line within each column with different letters are significantly different ($P < 0.05$).

The percentage inhibition of larval feeding after incubation with mimosa extract was significantly affected by concentration of CT ($P = 0.001$) but not by worm strain ($P = 0.984$). The feeding of the L1 was inhibited by 100% for all Mimosa extract concentrations from 200-1000 µg/mL for *H. contortus* susceptible, *H. contortus* MLs resistant and the *T. colubriformis* susceptible worm strains.

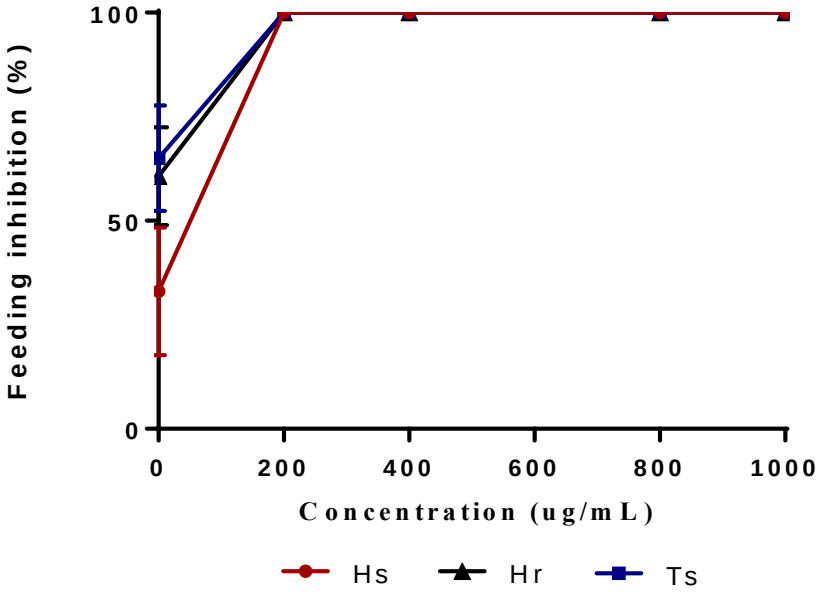


Figure 9.8 Means ± standard error of the means for feeding inhibition (%) of L1 from *H. contortus* susceptible (Hs), *H. contortus* MLs resistant (Hr) and the *T. colubriformis* susceptible (Ts) strains after incubation with mimosa extract.

Data for feeding inhibition after incubation with mimosa extract and pooled for worm strain are presented in Table 9.6 and Figure 9.9 below.

Table 9.6 Means ± standard error of the means for inhibition of larval feeding (%) for L1after incubation with mimosa extract, Thiabendazole, Ivermectin and DMSO. Data are pooled for worm strain.

Mimosa Extract (µg/mL)	**Feeding inhibition (%)**
0	51.36a ± 7.05
200	100.00b ± 0.00
400	100.00b ± 0.00
800	100.00b ± 0.00
1000	100.00b ± 0.00
P value	< 0.001
0	51.36a ± 7.05
0 + Thiabendazole	53.38a ± 25.16
0 + Ivermectin	100.00b ± 0.00
0 + DMSO	68.85a ± 42.36
P value	< 0.001

Rows above the dotted line within each column with different letters are significantly different (P < 0.05).
Rows below the dotted line within each column with different letters are significantly different (P < 0.05).

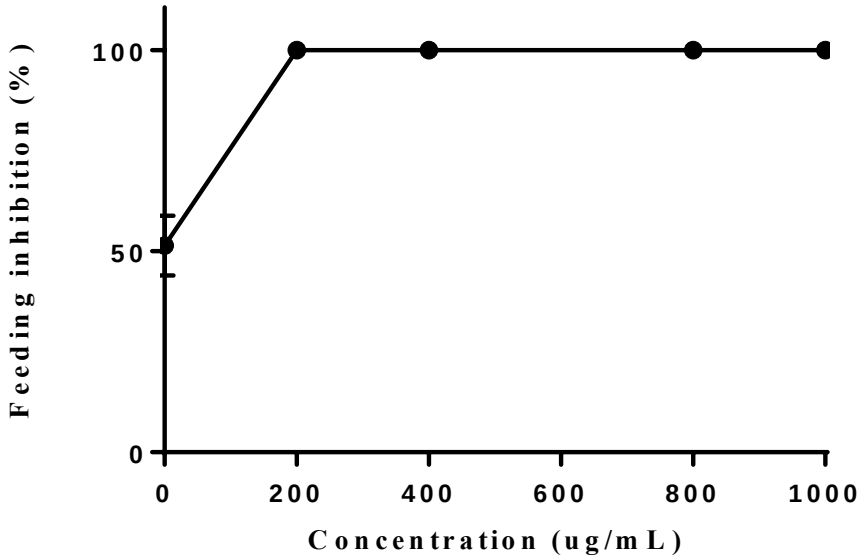


Figure 9.9 Means ± standard error of the means for inhibition of larval feeding (%) for L1 after incubation with mimosa extract. Data are pooled for worm strain.

The percent inhibition of larval feeding for the negative control (no added CT) pooled for worm strain was 51%. For the pooled data for worm strain there was a significant increase in the percent inhibition of larval feeding between the negative control and mimosa extract concentrations of 200-1000 µg/mL ($P < 0.001$). There were no significant differences ($P = 1.000$) in inhibition of larval feeding between mimosa extract concentrations of 200, 400, 800 and 1000 µg/mL.

The inclusion of DMSO as the solvent for the anthelmintic Thiabendazole was not different ($P = 0.413$) to the results for the negative controls of zero concentrations of mimosa extract for inhibition of larval feeding. The inclusion of the anthelmintic Ivermectin as the positive control had a significant effect on larval feeding compared to the negative controls ($P < 0.001$). Inhibition of feeding by Ivermectin was 100%. Thiabendazole was also used as another positive control, however, the inhibition of feeding was not different to the negative controls ($P = 0.495$).

9.4.5 Larval migration inhibition assays using calliandra condensed tannin

Data for larval migration inhibition using L3 of *H. contortus* susceptible, *H. contortus* MLs resistant and the *T. colubriformis* susceptible worm strains after incubation with calliandra CT are presented in Table 9.7 and Figure 9.10 below.

Table 9.7 Means ± standard error of the means for inhibition of larval migration (%) of L3 of *H. contortus* susceptible, *H. contortus* MLs resistant and the *T. colubriformis* susceptible strains after incubation with calliandra condensed tannin (CT), Levamisole, Ivermectin and DMSO.

Calliandra CT (µg/mL)	***H. contortus* (susceptible)**	***H. contortus* (resistant)**	***T. colubriformis* (susceptible)**
0	10.46 ± 3.39	45.56 ± 27.24	46.08 ± 26.98
200	69.01 ± 5.45	74.42 ± 12.32	31.73 ± 15.15
400	57.51 ± 8.24	73.92 ± 15.21	40.45 ± 14.35
800	42.94 ± 12.04	15.04 ± 3.20	53.42 ± 13.73
1000	79.70 ± 6.54	98.41 ± 1.59	67.64 ± 9.13
0	10.46 ± 3.39	45.56 ± 27.24	46.08 ± 26.98
0 + Levamisole	99.28 ± 0.72	93.35 ± 1.33	84.58 ± 2.78
0 + Ivermectin	99.21 ± 0.79	99.21 ± 0.79	99.21 ± 0.79
0 + DMSO	99.67 ± 0.33	99.67 ± 0.33	99.67 ± 0.33

Rows above the dotted line within each column with different letters are significantly different ($P < 0.05$).
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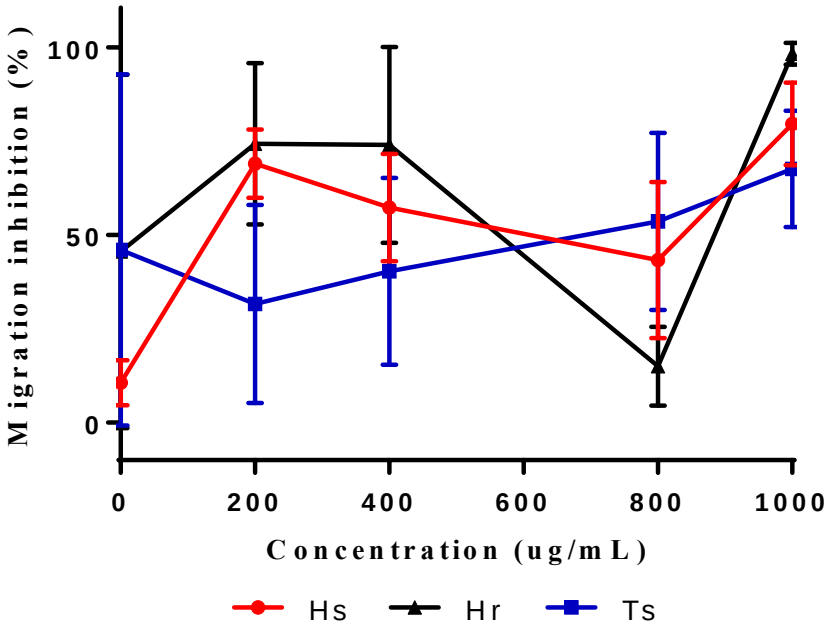

Figure 9.10 Means ± standard error of the means for inhibition of larval migration (%) of L3 of *H. contortus* susceptible (Hs), *H. contortus* MLs resistant (Hr) and the *T. colubriformis* susceptible (Ts) strains after incubation with calliandra condensed tannin.

The percentage inhibition of larval migration of L3 after incubation in calliandra CT was significantly affected by concentration of CT ($P < 0.001$) but not by worm strain ($P = 0.447$). Data for larval migration inhibition pooled for worm strain are presented in Table 9.8 and Figure 9.11 below.

Table 9.8 Means ± standard error of the means for inhibition of larval migration (%) of L3 after incubation with calliandra condensed tannin (CT), Levamisole, Ivermectin and DMSO. Data are pooled for worm strain.

Calliandra CT (µg/mL)	**Larval Migration Inhibition (%)**
0	34.03a ± 12.58
200	58.38a,c ± 8.90
400	57.29b,c,d ± 8.09
800	37.13a,d ± 7.99
1000	81.92b ± 5.55
P value	0.010
0	34.02a ± 19.20
0 + Levamisole	92.40b,c ± 1.61
0 + Ivermectin	99.21b ± 0.79
0 + DMSO	99.67b ± 0.33
P value	0.025

Rows above the dotted line within each column with different letters are significantly different ($P < 0.05$).
Rows below the dotted line within each column with different letters are significantly different ($P < 0.05$).

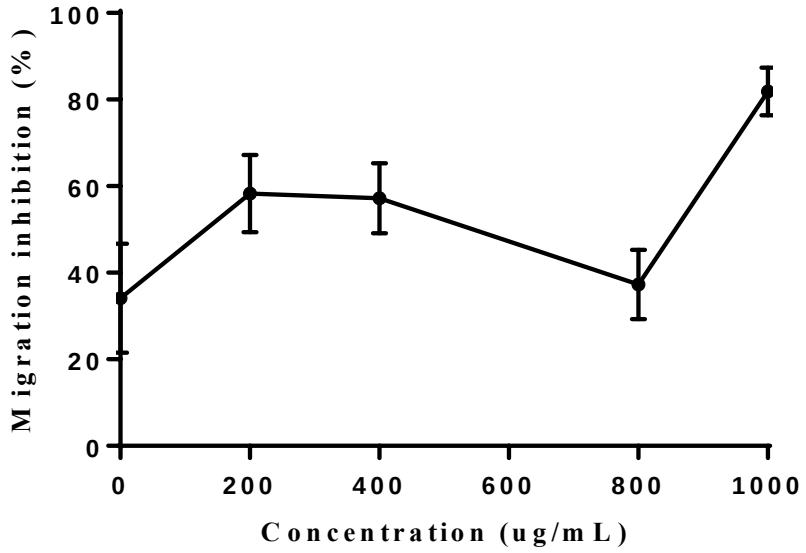

Figure 9.11 Means ± standard error of the means for inhibition of larval migration (%) of L3 of after incubation with calliandra condensed tannin. Data are pooled for worm strain.

The percent larval migration inhibition for the negative control (no added CT) pooled for worm strain was 34%. There was a significant increase in the inhibition of larval migration between the negative control and the calliandra CT at concentrations of 400 (P = 0.047) and 1000 (P = 0.030) µg/mL. The negative control was not different to the CT at concentrations of 200 (P = 0.171) or 800 µg/mL (P = 0.309). The percent inhibition of migration at a calliandra CT concentration of 200 µg/mL was not different to that of the 400 (P = 0.965) and 800 (P = 0.133) µg/mL, but was different to the 1000 µg/mL (P = 0.047). The percent inhibition of migration at a CT concentration of 400 µg/mL was not different to that of the 800 (P = 0.085) but different to 1000 µg/mL (P = 0.047). There was a difference (P = 0.002) between the CT concentrations of 800 and 1000 µg/mL for the percent inhibition of larval migration.

The negative control was significantly different for percent inhibition of migration from the anthelmintics, Levamisole (P = 0.030) and Ivermectin (P = 0.008), and the solvent DMSO (P = 0.008).

9.6.5.1 Relationship between percent migration inhibition and calliandra condensed tannin
The relationship between larval migration inhibition and calliandra CT concentration can be seen in Figure 9.12. A positive quadratic relationship was found to exist between percent migration inhibition and calliandra CT concentration (P = 0.019; r_s coefficient = 0. 348; r^2 = 0.097).

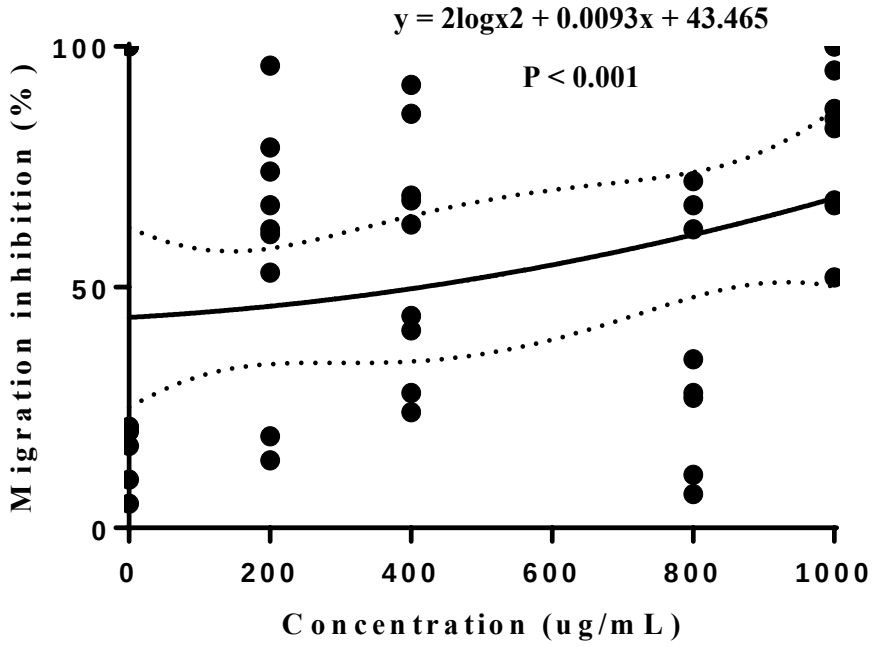

Figure 9.12 Relationship between percent inhibition of migration and calliandra condensed tannin concentration (µg/mL).

9.4.6 Larval migration inhibition using mimosa extract

Data for larval migration inhibition using L3 of *H. contortus* susceptible, *H. contortus* MLs resistant and the *T. colubriformis* susceptible worm strains after incubation with mimosa extract are presented in Table 9.9 and Figure 9.13 below.

Table 9.9 Means ± standard error of the means for inhibition of larval migration (%) for L3 of *H. contortus* susceptible, *H. contortus* MLs resistant and the *T. colubriformis* susceptible strains after incubation with mimosa extract, Levamisole, Ivermectin and DMSO.

Mimosa Extract (µg/mL)	***H. contortus* (susceptible)**	***H. contortus* (resistant)**	***T. colubriformis* (susceptible)**
0	10.46 ± 3.39	45.56 ± 27.24	46.08 ± 26.98
200	75.81 ± 0.97	86.46 ± 7.87	59.85 ± 22.47
400	74.33 ± 4.32	81.24 ± 1.74	17.72 ± 5.54
800	94.57 ± 2.88	97.71 ± 1.19	36.33 ± 21.50
1000	97.73 ± 0.13	78.39 ± 2.01	41.56 ± 27.62
0 + Levamisole	99.28 ± 0.72	93.35 ± 1.33	84.58 ± 2.78
0 + Ivermectin	99.21 ± 0.79	99.21 ± 0.79	99.21 ± 0.79
0 + DMSO	99.67 ± 0.33	99.67 ± 0.33	99.67 ± 0.33

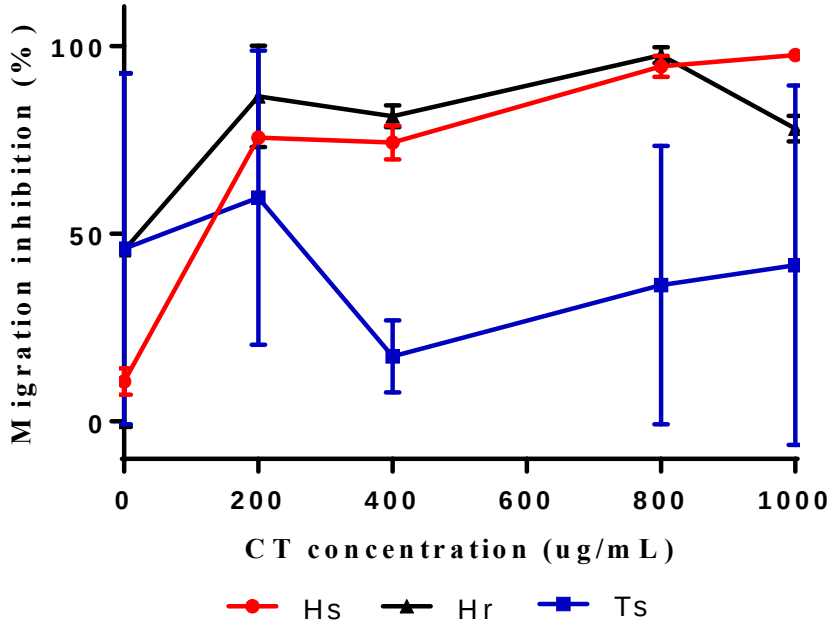

Figure 9.13 Means ± standard error of the means for inhibition of larval migration (%) of L3 of *H. contortus* susceptible (Hs), *H. contortus* MLs resistant (Hr) and the *T. colubriformis* susceptible (Ts) strains after incubation with mimosa extract.

The percentage inhibition of larval migration of L3 after incubation in mimosa extract was significantly affected by worm strain (P = 0.030) but not by concentration of mimosa extract

(P = 0.216). The *H. contortus* susceptible strain was not significantly different from the *H. contortus* MLs resistant strain (P = 0.885) or the *T. colubriformis* susceptible (P = 0.093) strain for percent migration inhibition. There was a significant difference in percent migration inhibition, however, between the *H. contortus* MLs resistant strain and the *T. colubriformis* susceptible (P = 0.009) strain.

9.4.7 Larval exsheathment assays using calliandra condensed tannin

Data for percent larval exsheathment and the time course for exsheathment (10-60 min) of L3 of *H. contortus* susceptible, *H. contortus* MLs resistant and the *T. colubriformis* susceptible worm strains incubated with calliandra CT are presented in Figures 9.14 A-C below.

Overall the results showed that there was a significant effect on exsheathment of L3 due to worm strain (P = 0.004), calliandra CT concentration (P = 0.001) and also time of exsheathment (P < 0.001).

The percent exsheathment of the *H. contortus* susceptible strain was different to that of the *H. contortus* MLs resistant strain (P = 0.004) but not different to that of the *T. colubriformis* susceptible strain (P = 0.926). There was a difference in percent exsheathment of the *H. contortus* MLs resistant strain compared to the *T. colubriformis* susceptible strain (P = 0.004).

There were significant differences in the overall data for percent exsheathment between the negative controls (no added CT) compared to the calliandra CT at 800 (P = 0.006) and 1000 µg/mL (P = 0.004). The data were also different between the calliandra CT concentartions of 200 µg/mL compared to both the 800 (P = 0.002) and 1000 µg/mL (P = 0.002) and also between the 400 and 800 µg/mL CT (P = 0.037). However, for percent larval exsheathment, there were no significant differences due to calliandra CT concentration within the individual worm strains, *H. contortus* susceptible (p = 0.257), *H. contortus* MLs resistant (P = 0.078) and *T. colubriformis* susceptible (P = 0.152).

Percent exsheathment was significantly different due to time of exsheathment for each individual worm strain (P < 0.001). For the *H. contortus* susceptible strain, the percent

exsheathment was significantly different between all times of 10, 20, 40 and 60 min ($P < 0.001$) and was different for each calliandra CT concentration at 10 min ($P = 0.026$), 20 min ($P = 0.011$) and 40 min ($P = 0.016$) but not different at 60 min ($P = 0.152$). There was a dose-dependant effect on time to maximum exsheathment, with the higher CT concentration having the effect of delaying exsheathment time for the *H. contortus* susceptible strain. Maximum exsheathment occurred for the PBS negative control and calliandra CT concentrations of 200 and 400 µg/mL between 20-40 mins, however, maximum exsheathment did not occur until 40-60min for the higher calliandra CT concentrations of 800 and 1000 µg/mL (Figures 9.14 A).

For the *H. contortus* MLs resistant strain, the percent exsheathment was significantly different between all times ($P < 0.001$) except 40 and 60 min ($P = 0.0.067$) and was different for each CT concentration at 20 min ($P = 0.021$), 40 min ($P = 0.011$) and 60 min ($P = 0.010$) but not different at 10 min ($P = 0.065$). There was a dose-dependant effect on time to maximum exsheathment, with the higher CT concentration of 800 and 1000 µg/mL having the effect of not only delaying exsheathment time for the *H. contortus* MLs resistant strain, but also inhibiting exsheathment. Maximum exsheathment was not achievd after 60 min. Maximum exsheathment occurred for the PBS negative control and CT concentrations of 200 and 400 µg/mL between 20-40 min, however, maximum exsheathment did not occur until 40-60 min for the higher CT concentrations of 800 and 1000 µg/mL (Figure 9.14 B).

For the *T. colubriformis* susceptible strain, the percent exsheathment was significantly different between times of 10, 20, 40 and 60 min ($P < 0.001$) and was different for each CT concentration at 10 min ($P = 0.014$), 20 min ($P = 0.024$) and 40 min ($P = 0.008$) but not different at 60 min ($P = 0.817$). There is a dose-dependant effect on time to maximum exsheathment, with the higher CT concentration having the effect of delaying exsheathment time for the *T. colubriformis* susceptible strain. Maximum exsheathment occurred for the PBS negative control and CT concentrations of 200 and 400 µg/mL between 20-40 min, however, maximum exsheathment did not occur until 40-60 min for the higher CT concentrations of 800 and 1000 µg/mL (Figure 9.14 C).

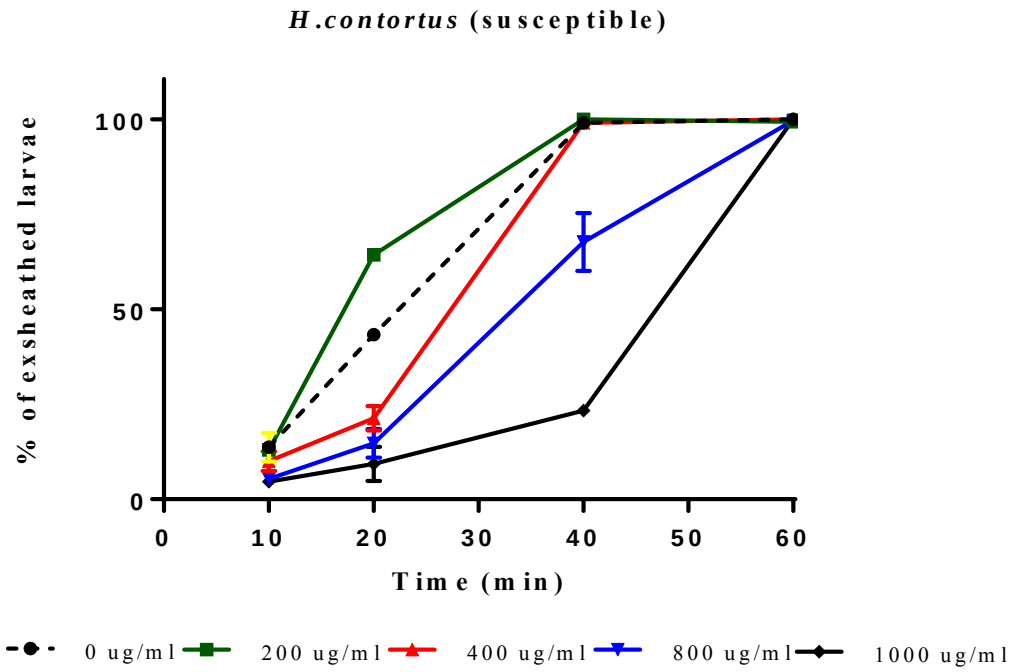

Figure 9.14 A Means ± standard error of the means for larval exsheathment (%) of L3 of *H. contortus* susceptible strain at 10, 20, 40 and 60 min after incubation with calliandra condensed tannin (µg/mL).

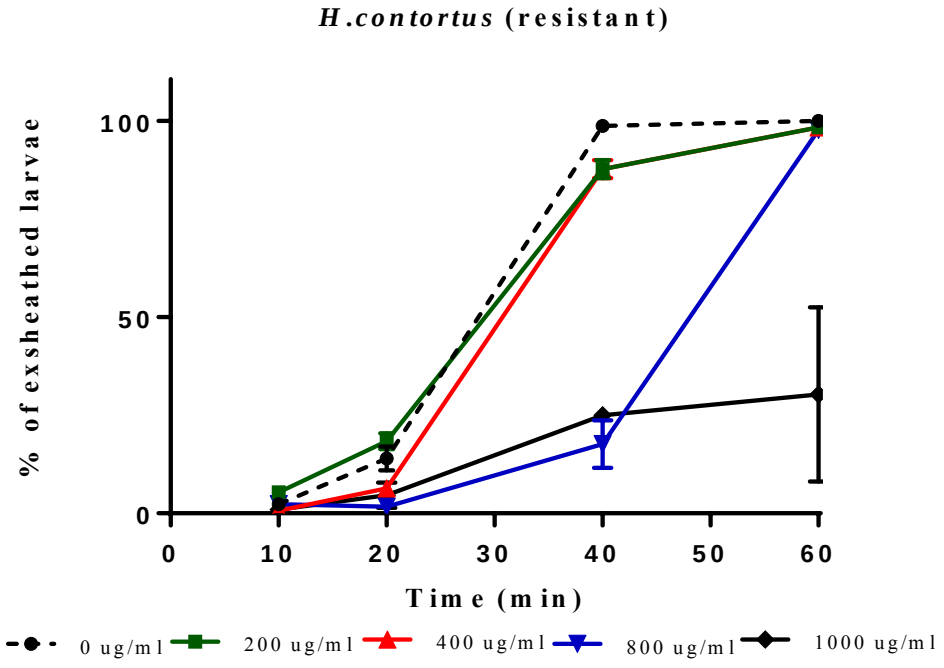

Figure 9.14 B Means ± standard error of the means for larval exsheathment (%) of L3 of *H. contortus* MLs resistant strain at 10, 20, 40 and 60 min after incubation with calliandra condensed tannin (µg/mL).

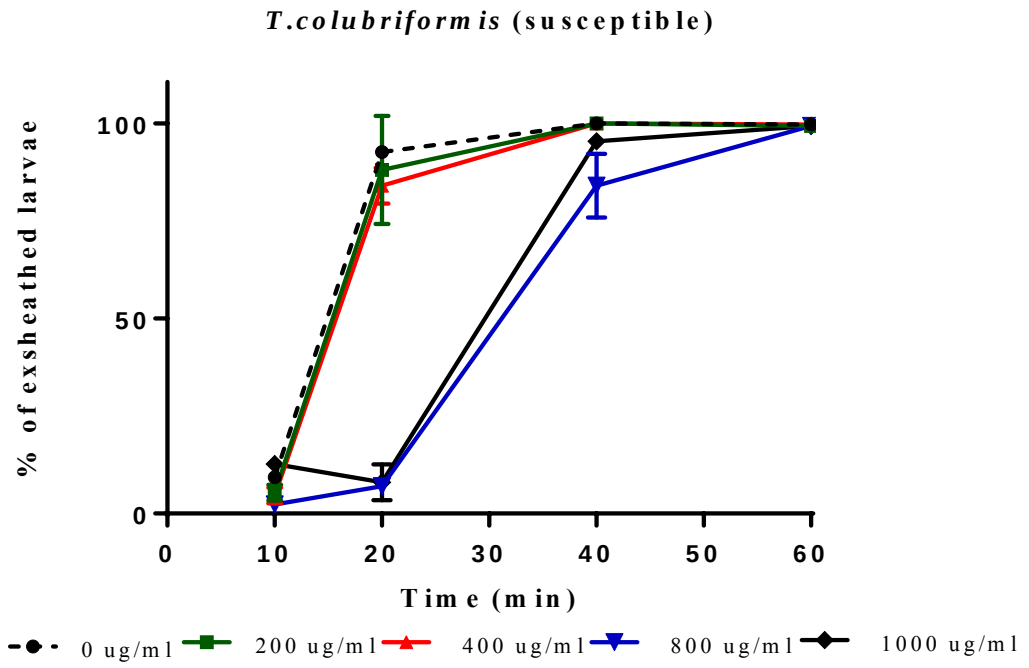

Figure 9.14 C Means ± standard error of the means for larval exsheathment (%) of L3 of *T. colubriformis* susceptible strain at 10, 20, 40 and 60 min after incubation with calliandra condensed tannin (µg/mL).

9.4.8 Larval exsheathment assays using mimosa extract

Data for percent larval exsheathment and the time course for exsheathment (10-60 min) of L3 of *H. contortus* susceptible, *H. contortus* MLs resistant and the *T. colubriformis* susceptible worm strains incubated with mimosa extract are presented in Figures 9.15 A-C below.

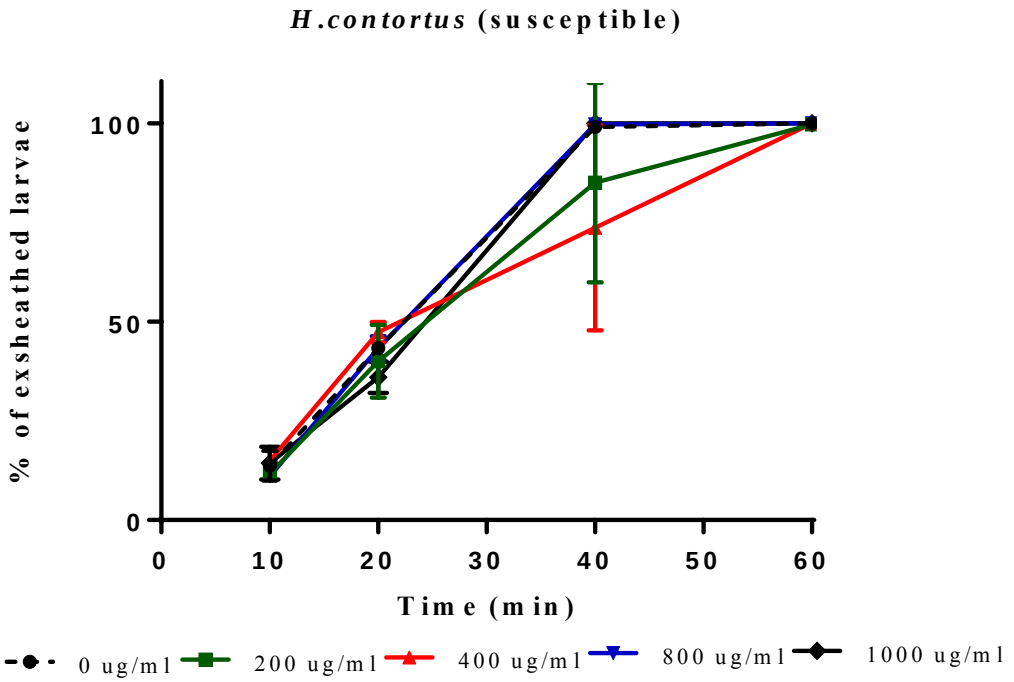

Figure 9.15 A Means ± standard error of the means for larval exsheathment (%) of L3 of *H. contortus* susceptible strain at 10, 20, 40 and 60 min after incubation with mimosa extract (µg/mL).

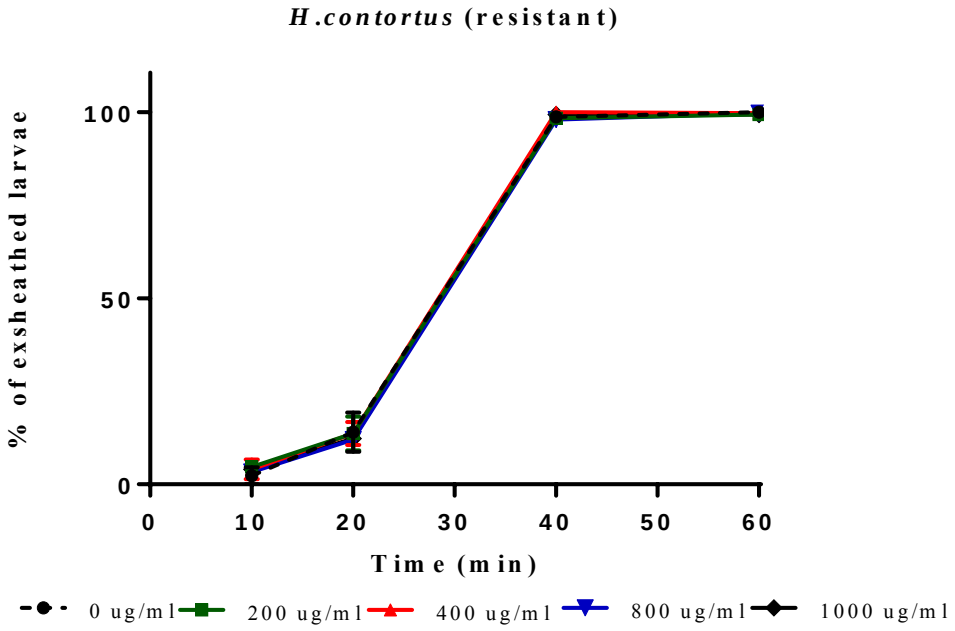

Figure 9.15 B Means ± standard error of the means for larval exsheathment (%) of L3 of *H. contortus* MLs resistant strain at 10, 20, 40 and 60 min after incubation with mimosa extract (µg/mL).

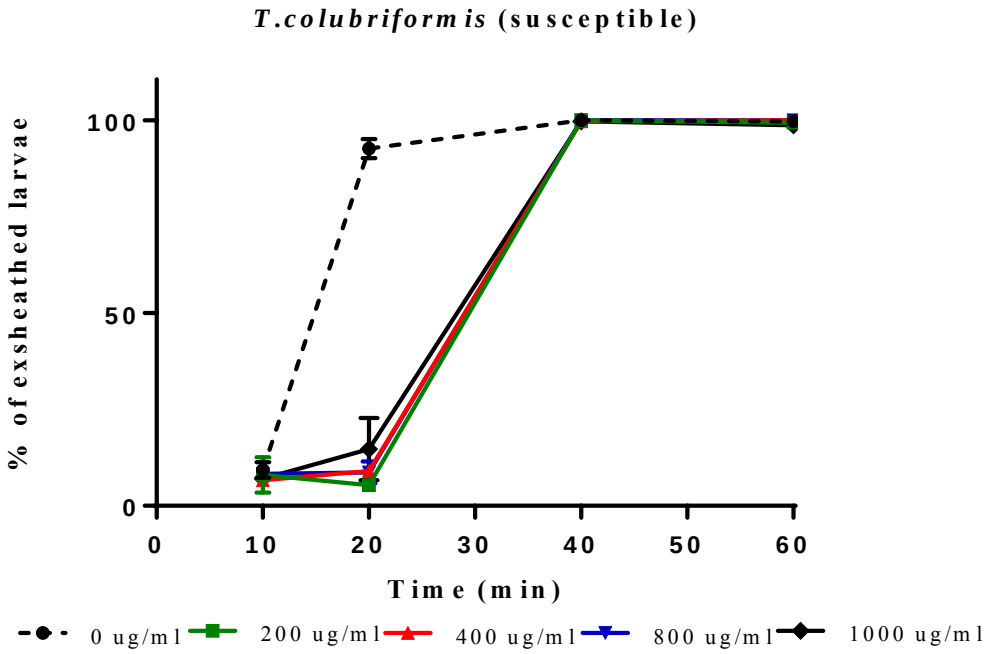

Figure 9.15 C Means ± standard error of the means for larval exsheathment (%) of L3 of *T. colubriformis* susceptible strain at 10, 20, 40 and 60 min after incubation with mimosa extract (µg/mL).

Overall the results showed that there was a significant effect on exsheathment of L3 due to worm strain ($P = 0.036$) and time of exsheathment ($P < 0.001$) but no difference due to mimosa extract concentration ($P = 0.882$).

Data for percent larval exsheathment and the time course for exsheathment (10-60 min) of L3 of *H. contortus* susceptible, *H. contortus* MLs resistant and the *T. colubriformis* susceptible worm strains pooled for mimosa extract concentration are presented in Table 9.10 and Figure 9.16 below.

Table 9.10 Means ± standard error of the means for larval exsheathment (%) of L3 of *H. contortus* susceptible, *H. contortus* MLs resistant and the *T. colubriformis* susceptible strains at 10, 20, 40 and 60 min pooled for mimosa extract concentration.

Time (min)	*H. contortus* (susceptible)	*H. contortus* (resistant)	*T. colubriformis* (susceptible)
10	12.97a ± 0.72	13.63a ± 0.53	7.85a ± 0.60
20	41.86b ± 1.51	13.23b ± 0.78	25.98a ± 0.98
40	99.42c ± 0.19	98.91c ± 0.28	99.85b,c ± 0.11
60	99.95c ± 0.05	99.57c ± 0.17	99.52b,c ± 0.16
P value	< 0.001	< 0.001	< 0.001

Rows within each column with different letters are significantly different (P < 0.05).

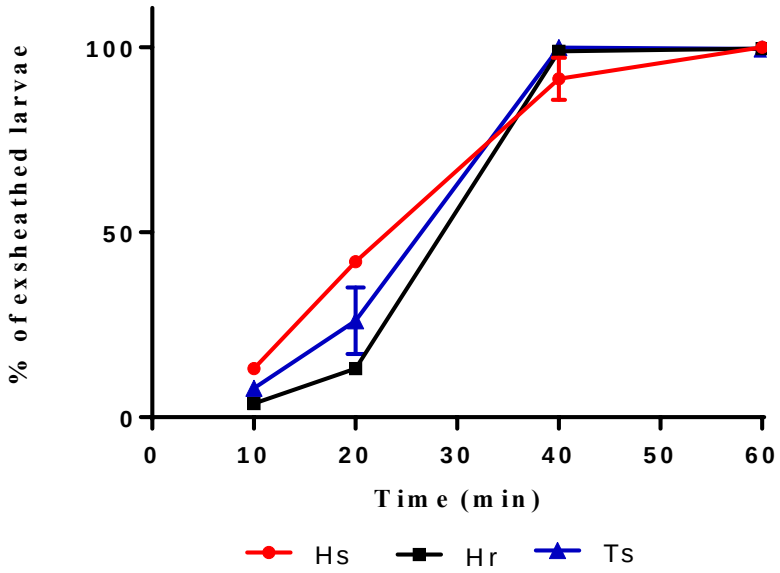

Figure 9.16 Means ± standard error of the means for larval exsheathment (%) of L3 of *H. contortus* susceptible (Hs), *H .contortus* MLs resistant (Hr) and the *T. colubriformis* susceptible (Ts) strains at 10, 20, 40 and 60 min pooled for mimosa extract concentration.

The percent exsheathment of the *H. contortus* susceptible strain was different to that of the *H. contortus* MLs resistant strain (P = 0.013) but not different to that of the *T. colubriformis* susceptible strain (P = 0.083). There was also no difference in percent larval exsheathment between the *H. contortus* MLs resistant strain compared to the *T. colubriformis* susceptible strain (P = 0.363).

Percent larval exsheathment was significantly different due to time of exsheathment for each individual worm strain ($P < 0.001$). For the *H. contortus* susceptible strain, the percent exsheathment was significantly different between all times of 10, 20, 40 and 60 min ($P < 0.001$, except at 40-60 min $P = 0.013$). For the *H. contortus* MLs resistant strain, the percent larval exsheathment was significantly different between all times ($P < 0.001$) except between 40 and 60 min ($P = 0.088$). For the *T. colubriformis* susceptible strain, the percent larval exsheathment was significantly different between all times of 10, 20, 40 and 60 min ($P < 0.001$) except between 10 and 20 min ($P = 0.120$) and between 40-60 min ($P = 0.069$).

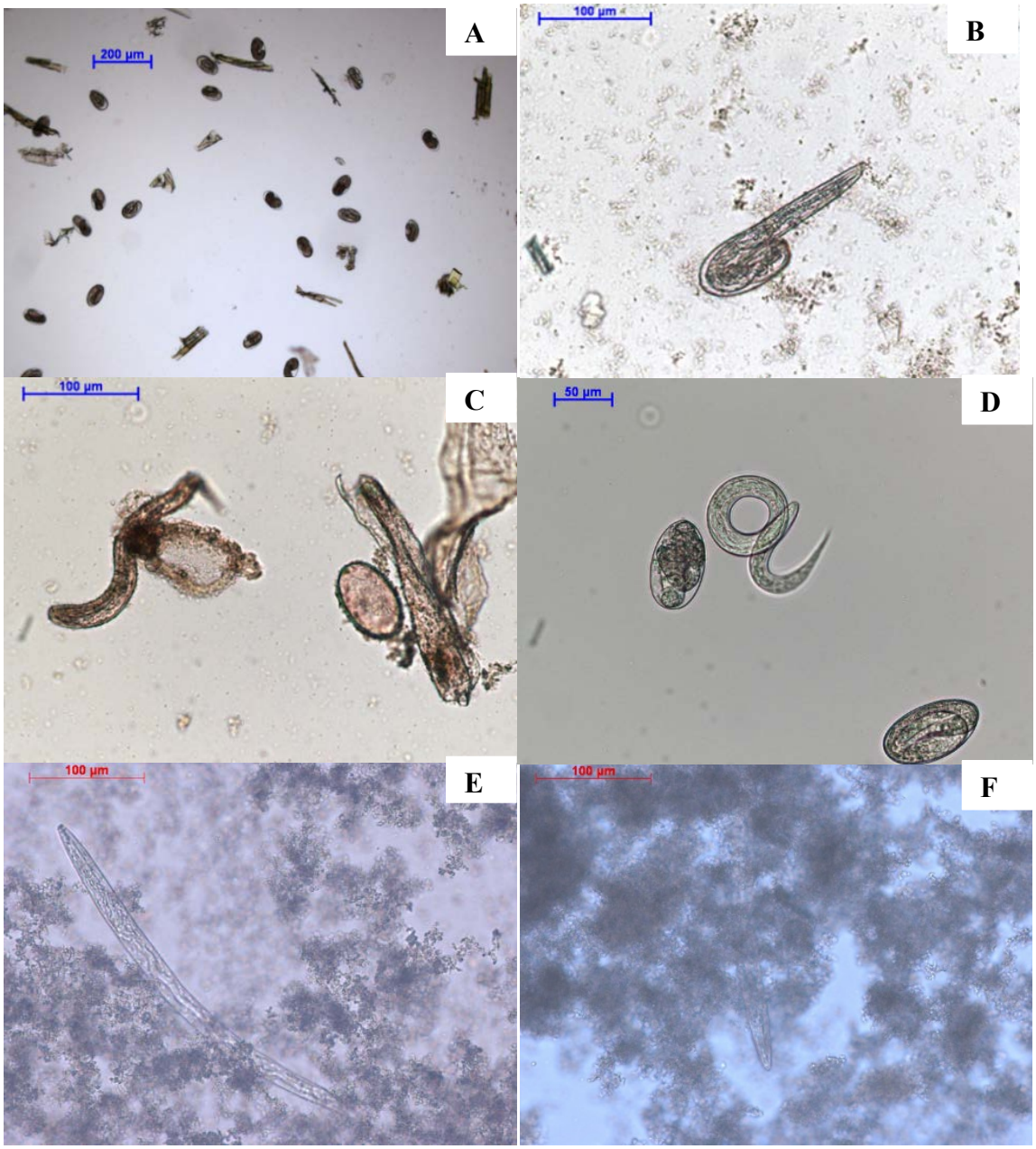

Plate 9.3 (A) *H. contortus* MLs resistant eggs before egg hatch assay showing advanced larvae development. (B) Partially hatched eggs from egg hatch assay with calliandra condensed tannin at 1000 µg/mL. (C) L1, an unhatched egg and debris from egg hatch assay with mimosa extract of 1000 µg/mL. (D) *T. colubriformis* L1 and unhatched eggs from the negative control of the egg hatch assay. (E) *T. colubriformis* L1 from the larval feeding inhibition assay with calliandra CT at 400 µg/mL in which CT can be seen to be clumping with the FITC labelled *E.coli*. (F) *T. colubriformis* L1 from the larval feeding inhibition assay with calliandra CT at 800 µg/mL in which CT can be seen to be clumping with the FITC labelled *E.coli.*

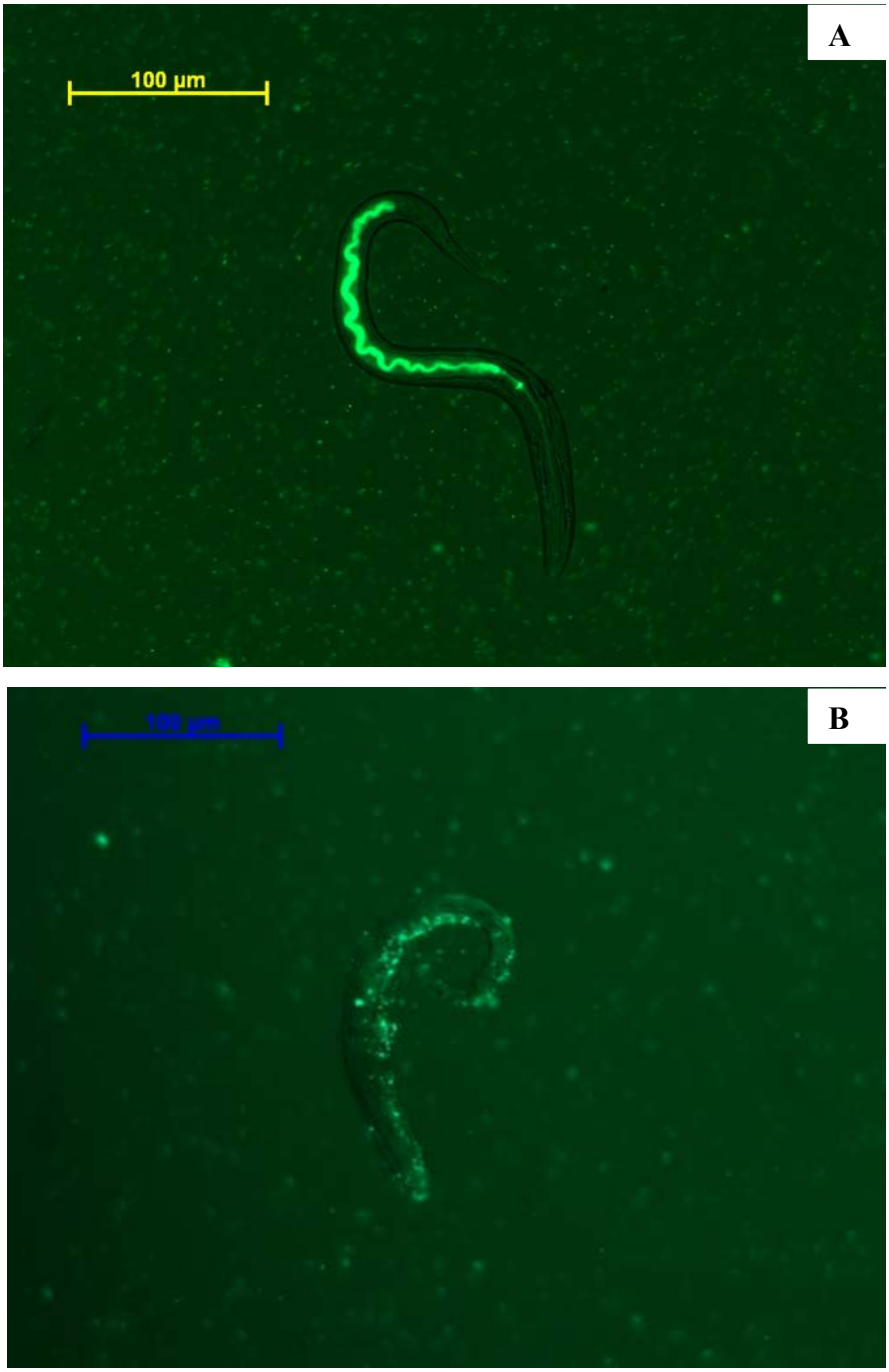

Plate 9.4 (A) Fed L1 for the negative control from the larval feeding inhibition, note the highly fluorescent gastrointestinal tract with no clumping of the FITC labelled *E.coli* in the background. (B) Unfed *H. contortus* MLs resistant L1 from the larval feeding inhibition, note no fluorescence in the gastrointestinal tract, however FITC labelled *E.coli* can be seen attached to the cuticle.

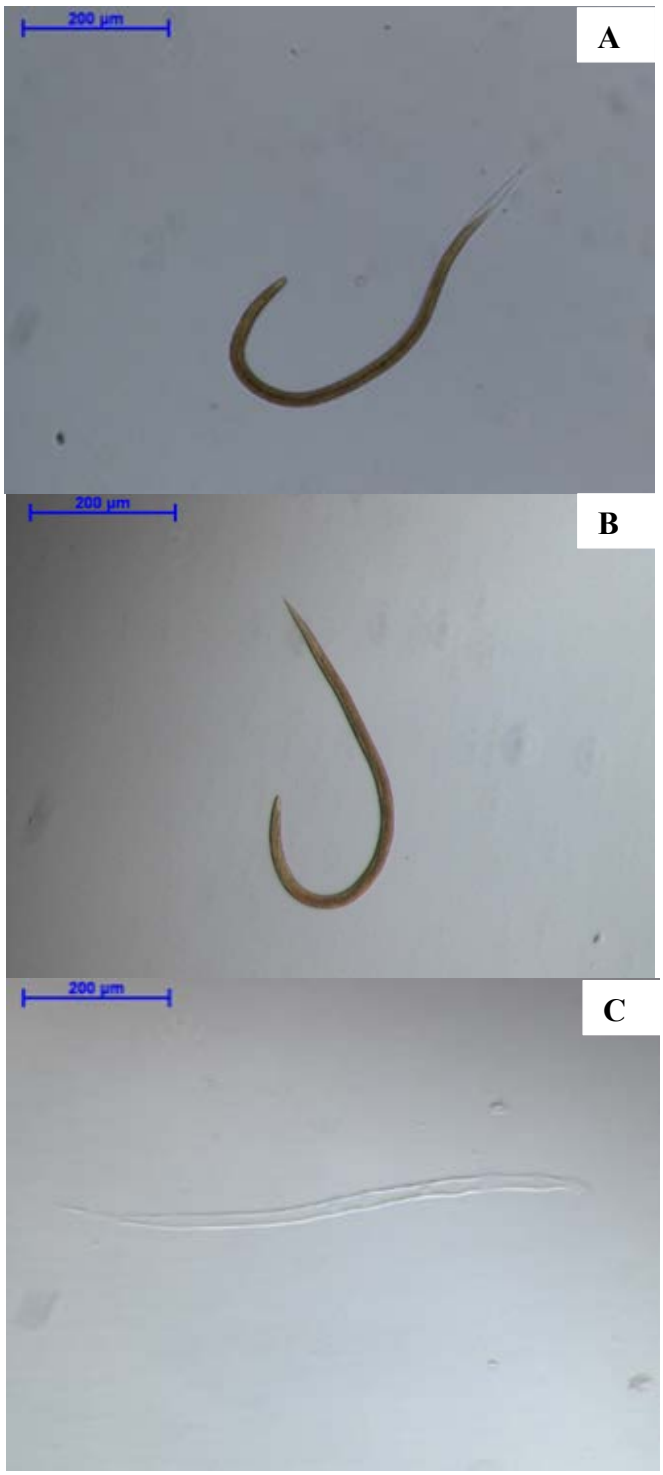

Plate 9.5 (A) Ensheathed L3 from the larval exsheathment assay. (B) Exsheathed L3 from the larval exsheathment assay (C) A free sheath from the larval exsheathment assay.

9.5 Discussion

This study shows that purified calliandra CT and mimosa extract are capable of disrupting different stages of the lifecycle *in vitro* of *H .contortus* susceptible, *H. contortus* MLs resistant strains and *T. colubriformis* susceptible strain. However, not all results from *in vitro* studies can be correlated *in vivo* due to the physiological conditions present in the GI tract and the interactions with constituents of digesta and secretions.

Calliandra CT had a greater effect than mimosa extract in the EHA, LEA and the LMIA. This study used concentrations of 200, 400, 800 and 1000 µg/mL of the purified calliandra CT (93.4% CT, as described in Chapter 3) and commercial mimosa extract from the bark of *Acacia mearnsii* (black wattle) which is sold as a tanning powder containing 68.3-72.3 % tannins. A difference in the LFIA may have been seen if experiments were conducted at lower tannin concentrations. The different tannin compositions and purity of the mimosa extract and the calliandra CT, and the fact that the tannin content is lower in the mimosa extract may account for the differences in the anthelmintic effects. The difference in the effect may also be attributed to the type of proanthocyanidin that is predominant in the plant as well as these ratios. The most common proanthocyanidins are procyanidin and prodelphinidin. The flavan-3-ols (monomer units of CT) are based on (-)-epicatechin and (+)-catechin polymers, which yield cyanidin and are therefore called procyanidins. The addition of a third phenolic group on the B ring produces gallocatechin and epigallocatechin based polymers, which yield delphinidin. Other less common proanthocyandins include prorobinetinidin, profisetinidin. There is also a rare mono-substituted flavan-3-ol based polymer, which yields pelargonidin. calliandra CT is made up of 82.6% procyanidin, 8.7% is prodelphinidin and only 2.2% is made up of propelargonidin (Rosales, 1999). *Acacia mearnsii* is predominately made up of prorobinetinidin, profisetinidin (Brown and Ho, 1997). Increases in the ratio of prodelphinidin: procyanydin have also been shown to influence protein complexing (Aerts et al. 1999). Monomers of prodelphinidin have been shown to have a greater effect of the exsheathment of infective larvae of *T. colobriformis* and *H. contortus* than monomers of procyanidin (Brunet 2006). Molan et al. (2003) used Flavan-3-ol and Flavan-3-ol galloyl derivatives in EHA, larval

development and LMIA in *T. colubriformis*. Molan et al. (2003) reported galloyl derivatives to have 100% inhibition of egg hatching at 1000 µg/mL compared with 20% inhibition of egg hatching with the Flavan-3 ols in *T. colubriformis*. Molan et al. (2003) also reported galloyl derivatives to also have approximately 35% inhibition larval migration at 500 µg/mL compared with approximately 20% inhibition of larval migration with the Flavan-3 ols in *T. colubriformis*. This was also the case for the larval migration study.

There were differences in the effectiveness of the calliandra CT and the mimosa extracts, with respect to anthelmintic effects due to the different worm strains for the EHA, LEA and the LMIA. Other workers have also reported species effects in *in vitro* assays (Paolini et al. 2004; Novobilský et al. 2011; Tibe et al. 2013).
With plant extracts PVPP or PEG, known to bind to and inactivate the biological properties of CT, are often used in *in vitro* assays to counteract and confirm the effects of active ingredient CT. The calliandra CT used in this current work was purified as described in Chapter 3, however, the mimosa extract was not, and therefore, experiments including either PVPP or PEG would determine if any of the effects were due to CT, other plant secondary metabolites.

9.5.1 Egg hatch assays

The EHA determines the direct effects of tannins on disrupting the process of egg hatching. If hatching is inhibited the result will be a decrease in the number of L1 on pasture. In this current study calliandra CT and mimosa extract concentrations of 200, 400, 800 and 1000 µg/mL were used. These concentrations are well below those found in the GI segments of the lambs on a 100% calliandra diet reported in Chapter 5. The *H. contortus* females would start producing eggs in the abomasum where the CT concentration in abomasal digesta would be 53 000 µg/mL and the *T. colubriformis* adults would start producing eggs in the duodenum where the CT concentration in duodenal digesta would be 9 000 µg/mL. The eggs of both species would then be exposed to digesta in ileum, caecum and colon with CT concentrations of 26 000 µg/mL, 30 000 µg/mL and 31 000 µg/mL, respectively. The eggs would then remain on pasture in the faeces with a CT concentration of 28 000 µg/mL for

many days. For lambs fed a 100% calliandra diet, the eggs of both worm species would be exposed to higher concentrations of tannins and for a longer period of time from being laid in the GI tract, to hatching in the faeces in the pasture and would, therefore, have a greater effect on egg hatching.

With purified calliandra CT the *H. contortus* susceptible strain had the least eggs hatched at 53% compared to the *H. contortus* MLs resistant strain at 71% and the *T. colubriformis* susceptible strain at 70% for 1000 µg/mL CT. The dose-response of egg hatching to the calliandra CT was only significant for the *H. contortus* susceptible and the *T. colubriformis* susceptible strains. There was a similar result for the mimosa extract with respect to the *H. contortus* susceptible strain with egg hatch at 51% at 1000 µg/mL mimosa extract and also a significant dose-response of egg hatching to mimosa extract. The mimosa extract did not have any effect on egg hatching for the *H. contortus* MLs resistant or the *T. colubriformis* susceptible strains. The greater response to the egg hatching found in the calliandra CT compared to the mimosa extract is undoubtably due to the differences in the type, purity and concentration of tannins.

Results from other studies (Athanasiadou et al. 2001; Molan et al. 2002; Molan and Faraj 2010; Marie-Magdeleine et al. 2010) have shown variable responses in the EHA depending on the nematode species and the CT plant extract tested. Quebracho extract was found to have no effect on egg hatch of eggs of *H. contortus, T. circumcincta* and *T. vitrinis* (Athanasiadou et al. 2001). This was also the case for *Manihot esculenta* leaf extracts with no effect on egg hatching for *H. contortus* (Marie-Magdeleine et al. 2010). *T. circumcincta* eggs exposed to extracts from *Lotus pedunculatus, Lotus corniculatus, Dorycnium pentaphyllum, Dorycnium rectum,* and *Rumex obtusifolius* at CT concentration of 50-900 µg/mL all showed a decrease in egg hatch with increasing CT concentrations (Molan et al. 2002). Similar findings were also published by Molan and Faraj (2010) using the same nematode and plant extracts but at 900 µg/mL CT with variable egg hatch decrease of 53% for *Lotus pedunculatus,* 68% for *Lotus corniculatus,* 51% for *Dorycnium pentaphyllum,* 60% for *Dorycnium rectum,* and 46% for *Rumex obtusifolius* which are similar results to that reported in chapter 9.

The *H. contortus* susceptible strain in this study had a greater percentage inhibition of eggs hatched compared to the *H. contortus* MLs resistant and the *T. colubriformis* susceptible strains. This may be explained by stage of egg development at the time the assay commenced or that these *H. contortus* MLs resistant and the *T. colubriformis* susceptible strains are less susceptible to the effects for calliandra CT and mimosa extract. Upon arrival of *H. contortus* susceptible strain eggs at James Cook University from the commercial supplier, there was hardly any larval development. For the *H. contortus* MLs resistant and the *T. colubriformis* susceptible strains, however, on arrival the eggs were found to be in advanced larval development. The eggs of the *H. contortus* MLs resistant and the *T. colubriformis* susceptible strains would have had exposure to the calliandra CT and mimosa extracts for a shorter period before hatching commenced. Unfortunately, it was not possible to wait for another order of eggs, however, it would be desirable to repeat the EHA with all eggs at the same stage of development.

The structure of nematode egg shells is very complex with most species having the egg shells made up of three layers secreted by the fertilised oocyte (Wharton 1980). The egg-shell consists of an outer vitelline layer, a chitin/protein complex and an inner lipid layer. The chitin micro fibrils are surrounded by a protein coat (proline being the major amino acid) to form a composite fibril (Wharton 1980) and the inner lipid layer is the main permeability barrier.

The exact mechanisms by which egg hatching is inhibited by CT are unknown. The CT may be interacting with the proline rich protein on the surface of the egg. There is evidence to suggest that the bonding between CT and protein is strengthened when the amide nitrogen adjacent to the carbonyl is alkyl substituted (Hagerman 1980), as with the imino nitrogen in the peptide linkage with proline. The chitin/protein composite is highly resistant to environmental hazards and the mechanical and chemical resistance may be further increased by tanning (Wharton 1983). Egg hatching in the faeces occurs due to environmental stimuli which produces the release of “hatching enzymes” (Sommerville and Rogers 1987), including chitinases, proteases, lipases, leucine aminopeptidases and beta-glycosidases. It has been

demonstrated that the inhibition of some of these enzymes reduce egg hatching (Rogers and Brooks 1977). It has been shown in Chapter 8 that calliandra CT is a potent inhibitor of the digestive enzymes, trypsin and pancreatic α-amylase at very low CT concentrations. The inhibition of digestive enzymes has also been demonstrated by other workers (Gu et al. 2001; Kazeem et al. 2013; Horigome et al. 1988). It is possible that the enzymes released during the hatching process may also be inhibited by calliandra CT and mimosa extract also.

9.5.2 Larval feeding inhibition assays

Larval feeding inhibition assays target the feeding behaviour of L1. If feeding is disrupted it will potentially kill the L1 by starvation and will have the effect of less L1 being able to develop into infective L3 on pasture.

The results of this LFIA were quite clear with all calliandra CT and mimosa extract concentrations tested (200-1000 µg/mL) having 100 % inhibition of feeding on the *H. contortus* susceptible, *H. contortus* MLs resistant and the *T. colubriformis* susceptible strains. There were no significant differences between the three worm species for inhibition of larval feeding inhibition for the calliandra CT or the mimosa extract. In the negative controls mean feeding inhibition was 34% for the larvae exposed to calliandra CT and 51% for the assay using the mimosa extract. Most of the studies reported in the literature for LFIA use commercial anthelmintics such as MLs and imidazothiazole to test efficacy of these drugs on different worm strains (Álvarez-Sánchez et al. 2005). There are few studies reported in the literature using LFIA to screen the effects of plant extracts on nematodes. Bartley et al. (2009) have reported larval feeding inhibition in sheep nematodes exposed to 119 of the 513 plant extracts screened. The other study published by Novobilský et al. (2011) is also in agreement with the results of this chapter reporting 100% larval feeding inhibition at 160 µg/mL CT concentration using extracts from *Lotus pedunculatus, Lotus corniculatus* and *Onobrychis viciifolia* for *C. oncophora* from calves. The percent inhibition of feeding in this same study was lower with the *O. ostertagia* for all plant extracts at >95%.The mean feeding inhibition for the negative controls are also

similar to that found in chapter 9 at 39% and 43% for the *C. oncophora and O. ostertagia,* respectively.

The L1 hatch, feed and develop to the next stage in the faeces on pasture. The CT concentrations tested (200-1000 µg/mL) are well below what is found in the faeces of lambs fed a 100% calliandra diet (Chapter 5), at 28 000 µg/mL. Our data shows that feeding inhibition of L1 is very sensitive to very low (< 200 µg/mL) CT concentrations.

Death by starvation is the obvious consequence of feeding inhibition, however, different mechanisms of action for the inhibition of L1 feeding have been proposed in the literature. Different classes of anthelmintics have different modes of action. Geary et al. (1993) proposed that Ivermectin-induced paralysis of the pharynx was responsible for significant larval feeing inhibition in the five L1that were tested from *H. contortus*. Other workers have reported that the inhibition of larval feeding due to exposure to MLs appears to be caused by flaccid paralysis of the pharynx (Gill et al. 1995; Martin 1996). Sangster (1996) reported that inhibition of larval feeding was due to a generalised spastic paralysis of the muscles of the L1 when exposed to Imidazothiazoles. The study by Novobilský et al. (2011) also reported whether the larvae were dead or alive and found blisters between cuticle and larvae body in some live and unfed larvae. This is also in agreement with Bartley et al (2009) with 14 of the tested plant extracts also producing cuticle blistering. The experimental work in this chapter did not distinguish between dead and alive larvae as all larvae were preserved with formalin before counting.

Initial test assay (results not reported here) using *H. contortus* susceptible strain only at 100 -1000 µg/mL showed 100% inhibition with calliandra CT but only 85% inhibition with mimosa extract at 100 µg/mL, again supporting the lower efficacy of mimosa extract compared with calliandra CT that was also found in the EHA. It would be of value to repeat this experiment using lower concentration (< 100 µg/mL) of the calliandra CT and the mimosa extracts as well as to distinguish between dead and live L1 and to determine if any cuticle blistering had occurred.

9.5.3 Larval migration inhibition assay

Larval migration inhibition assays are used to determine if the test substance has the ability to inhibit motility of the L3 and therefore have the potential to prevent host infection by inhibiting movement of L3 from faeces to pasture. The assay may be conducted with either ensheathed or exsheathed (after artificial exsheathment) L3. L3 developing in faeces containing CT may not have the ability to move out of the faeces and onto pasture due to paralysis. The effect of this will be a decreased number of infectious L3 on pasture and therefore decreased infection rate.

The purified calliandra CT had a significant effect on L3 migration but the mimosa extract had no effect. The difference in effect may again be due to the differences in purity, type and concentration of CT found in the purified calliandra CT compared with the mimosa extract.

The results for this assay were quite variable (Figures 9.10 and 9.13) and may have been made statistically significant with a higher number of replicates. With the calliandra CT, however, there were no differences in the percent larval migration inhibition between *H. contortus* susceptible, *H. contortus* MLs resistant and the *T. colubriformis* susceptible strains with 81.92% percent inhibition of migration at 1000 µg/mL. Pooled data for worm strain showed a moderate dose-response in inhibition of larval migration due to calliandra CT. Although the mimosa extract did not have any significant effects on L3 migration there were overall differences between worm strains.

The percent larval migration inhibition for the negative controls (no added CT) were 10.46% for *H. contortus* susceptible, 46.56% for *H. contortus* MLs resistant and 46.08% for *T. colubriformis* susceptible worm strains. This is a variable result but was not significant due to the very large standard errors for the *H. contortus* MLs resistant and *T. colubriformis* susceptible strains. The mean percent larval migration inhibition for the negative controls was 34.5%. Other studies have varied reports of percentages for the negative control for larval migration inhibition for *H. contortus* of 7% (Manolaraki et al. 2010), 2-20% (Barrau et al. 2005), 0% (Tibe et al. 2013) and 42% (Paolini et al. 2004). Larval migration inhibition for controls using *T.*

colubriformis and *T.circumcinta* have be reported to be 30% and 33%, respectively (Paolini et al. 2004).

Results from other studies show variations in migration inhibition due to the type of plant extract and also worm species. Lotus, sainfoin and sulla were found to all result in inhibition of L3 migration (Molan et al. 1999; Molan et al. 2000a; Molan et al. 2000b). Molan et al. (2000a) tested the L3 from *T. colubriformis*, *H. contortus* and *O.circumcincta* exposed to sulla (*Hedysarum coronarium*) extract containing CT and found decreased larval migration with the *T .colubriformis* being the least affected. In another LMIA study (Molan et al. 2000b), it was reported that CT extracts from *L. pedunculatus, L. corniculatus,* sulla (*Hedysarum coronarium*) and sainfoin (*Onobrychus viciifloia*) had an inhibitory effect on L1 and L3 of deer lungworm (*Dictyocaulus viviparus*) and on L3 (51-77% inhibition) of deer GI nematodes. Molan et al. (2000b) also compared the effects of CT on the exsheathed and ensheathed larvae, with CT having the greater inhibitory effect on the exsheathed larvae. Manolaraki et al. (2010) tested the effect of extracts from eight Mediterranean browse plants on larval migration of *H. contortus* L3. Most of the plants caused a reduction in migration, with percent inhibition of larval migration of 79.7% for *Pistacia lentiscus,* 47.7% for *Pyrus spinosa,* 63.8% for *Quercus coccifera, Ceratonia silique, Olea europaea,* 54.6% for *Castanea sativa,* 61.4% and 61.5% *Ceratonia silique* fruit and leaves, respectively, and 38.9% for *Onobrychis viciifolia*. There was a dose-dependent effect of the plant extract for larval migration inhibition for *Quercus coccifera, Ceratonia silique fruit and leaves, Castanea sativa* and *Pyrus spinosa.* In a separate study conducted by Barrau et al. (2005) a significant inhibitory effect on migration of *H. contortus* L3 occurred due to exposure to sainfoin extracts. The degree of inhibition with sainfoin (30-70%) was also shown to depend on the type of solvent used in the plant extraction process.

Larval migration can be different in different worm species exposed to the same plant extracts. This is highlighted in the study using L3 from *H. contortus, T. colubriformis* and *Teladorsagia circumcincta* and extracts of *Onobrychis viciifolia, Quercus robur* and *Corylus Avella* were used (Paolini et al. 2004). There was inhibition of migration for *Corylus avellana* of 51% for *H. contortus,* 55.75% for *T .*

colubriformis and 55.5% for *Teladorsagia circumcincta.* There was also inhibition of migration for *Quercus robur* of 70.4% for *H. contortus,* 61.75% for *T. colubriformis* and 66.5% for *Teladorsagia circumcincta.* A greater variation was seen between the worm species for inhibition of migration due to the plant extract of *Onobrychis viciifolia* of 54% for *H. contortus,* 61.75% for *T. colubriformis* and 42% *Teladorsagia circumcincta.*

Similar results were found for mimosa extract with no inhibition of larval migration due to the effects of *Manihot esculenta* leaf extracts on *H. contortus* L3 at concentrations of 150-2400 µg/mL (Marie-Magdeleine et al. 2010). This is also in agreement with results of 0% larval migration inhibition found with 500 µg/mL of purified extracts from *Viscum rotubdufolium* and *Viscum verrucosum* (Tibe et al. 2013).

The CT concentrations tested (200-1000 µg/mL) are well below what is found in the faeces of lambs fed a 100% calliandra diet (Chapter 5), at 28 000 µg/mL. It is proposed that the L3 are very sensitive to calliandra CT but not to mimosa extracts.

The mechanism by which inhibition of migration of the L3 is presumably paralysis of the musculature but it is not well understood. The CT can potentially form complexes with sheath proteins but the L3 does not feed therefore it is not affected by ingested CT.

The data are quite variable, sometimes with large standard errors due to too much variation. Results would have been clearer if a greater number of replicates had been used. However, not ideal scientifically, three replicates were chosen due to time constraints. The method of Jackson and Hoste (2010) count the L3 that migrated into wells and also wash off what is remaining on the filter, however, it was noted that some L3 were physically embedded in the mesh of the filter so were neither above nor below it. Number of L3 embedded were not counted. It was initially thought that the tannins may be blocking some of the pores of the mesh filter, but Rabel et al. (1994) also reported variation in migration due to different filters and also due to pipetting. The pipette tips had their ends cut off so as to increase the diameter to

ensure no L3 were prevented from entering the pipette tip. Rabel et al. (1994) also found no differences in L3 migration numbers if half the mesh filters were blocked with parafilm or tape compared to filters that were not blocked.

9.5.4 Larval exsheathment assay

Larval exsheathment assays target the process of exsheathment of L3 induced by a dilute solution of sodium hypochlorite. If after L3 ingestion by the host, exsheathment is inhibited, death of the L3 will result or if the exsheathment is delayed, a decrease in establishment will occur because of the passage of the L3 to next GI segment. This process is a key biological step in the change from the infective stage to the parasitic phase (Hoste et al. 2015).

Calliandra CT was found to have an inhibitory effect on larval exsheathment, however, the mimosa extract was found to have no effect on L3 exsheathment.

The calliandra CT had a dose-dependant effect on time to maximum exsheathment, with the higher CT concentrations of 800 and 1000 µg/mL having the effect of delaying exsheathment time for all three worm strains. Not only was there a delay in time to maximum exsheathment, but there was a 70% inhibition of exsheathment at the highest CT concentration of 1000 µg/mL for the *H. contortus* MLs resistant strain after 60 min.

With the *H. contortus* susceptible strain maximum exsheathment of 100% for the negative control occurred between 20-40 min. Maximum exsheathment of 98% and 100% for the 800 and 1000 µg/mL CT concentrations did not occur until 40-60 min.

With the *H. contortus* MLs resistant strain maximum exsheathment of 100% for the negative control occurred between 20-40 min. Maximum exsheathment of 98% and 30% for the 800 and 1000 µg/mL CT concentrations did not occur until 40-60 min after incubation with exsheahment fluid. The highest CT concentration delayed and inhibited exsheathment.

With the *T. colubriformis* susceptible strain maximum exsheathment of 100% for the negative control occurred between 20-40 min. Maximum exsheathment of 99% and 99% for the 800 and 1000 µg/mL CT concentrations did not occur until 40-60 min.

Similar dose-dependent effects of CT concentration on inhibition of larval exsheathment have been reported (Bahuaud et al. 2006; Brunet et al. 2007; Novobilský et al. 2011; Aissa et al. 2015). Bahuaud et al. (2006) found variable results with respect to percent larval exsheathment of both *H. contortus* and *T. colubriformis* L3 with extracts of *Sarthamnus scoparius, Pinus sylvestris, Erica reigena* and *Castanea sativa* at 600 µg/mL CT. The CT concentrations of the plants were reported to be 0.03%, 2.5%, 19% and 24.7% DM for the *Sarthamnus scoparius, Pinus sylvestris, Erica reigena* and *Castanea sativa,* respectively. No effects were found on inhibition of larval exsheathment in the *Sarthamnus scoparius* for either worm species, probably due to the low CT (0.03%) content of the extract. There was an effect of delayed exsheathment for both worm species exposed to *Pinus sylvestris* plant extracts, and was also delayed for the *H. contortus* with the plant extract *Erica reigena,* but no effect was found to occur with the *T. colubriformis*. The plant extract with the highest CT concentration, *Castanea sativa* resulted in almost total inhibition of exsheathment for both *H. contortus* and *T. colubriformis.* Novobilský et al. (2011) reported inhibition of larval exsheathment of L3 from *C.oncophora* and *O.ostertagi* from calves incubated with CT from *O. vicifloia, L. pedunculatus* and *L. corniculatus* Novobilský et al. (2011) reported PBS controls as having exsheathment rates of 95% and 97% for *C. oncophora* and *O. ostertagi*, respectively, after 60 min of exposure to the artificial exsheathment fluid. Delays in exsheathment were similar to those reported in chapter 9 with differences in maximum larval exsheathment rates between the plant extracts *O. vicifloia* (50-55%), *L. pedunculatus* (60-65%) and *L. corniculatus* (70%) for *C. onophora* and *O. ostertagi* at CT concentrations of 2400 µg/mL after 60 min. The time course of exsheathment was also reported to be faster for the *O. ostertagi.* Although PBS controls were similar at 80% exsheathment, Aissa et al. (2015) reported maximum exsheathment of 2.67% for fresh sulla after 60 min for CT concentrations of 1200 µg/mL for L3 of *H. contortus*. This is a much greater effect than that found in the present study. There are also reports for both *H. contortus* and *T. colubriformis* having very high rates of inhibition of larval

exsheathment, with exsheathment of only 0-11% when exposed to extracts from the tropical plants *Lisyloma latisiliquum, Leacana leucocephala, Acacia pennatula* and *Piscidia piscipula* (Alonso-Diaz et al. 2008a; Alonso-Díaz et al. 2008b). The CT appear to be of higher concentrations than in this present study but it is difficult to compare when extracts were analysed with the vanillin assay and not the Butanol-HCl assay. Brunet et al. (2007) have also reported very high levels of inhibition of exsheathment of *H. contortus* and *T. colubriformis* L3 with exposure to extracts of sainfoin resulting in larval exsheathment of 11% and 0%, respectively, after 60 min at a CT concentration of 1200 μg/mL. *In vivo* assays were also conducted by Brunet et al. (2007) in conjunction with the *in vitro* LEA and found delayed larval exsheathment of both *H. contortus* and *T. colubriformis* when both were placed via a cannula into the rumen with saifoin. In contrast, Cresswell (2007) found no effects of decreased establishment of *H. contortus* or *T. colubriformis* in lambs fed 100% calliandra diet compared to a non-tannin diet.

The concentration of mimosa extract had no effect on percent exsheathment of L3. Greater than 99% exsheathment was achieved for the *H. contortus* susceptible, *H. contortus* MLs resistant and the *T. colubriformis* susceptible strains at 60 min after exposure to the artificial exsheathment fluid for data pooled for mimosa extract.

Again the concentration of calliandra CT and mimosa extracts used in the LEA were 200, 400, 800 and 1 μg/mL. The L3 initially inhabit the faeces on pasture, thereafter, ingestion by the ruminant occurs and the L3 of *H. contortus* and *T. colubriformis* exsheath in the rumen and abomasum, respectively. The tannin concentrations used in the LEA are well below the concentrations observed in Chapter 5 for lambs fed 100% calliandra diet in faeces, rumen and abomasal digesta at calliandra CT concentrations of 28 000, 19 000 and 53 000 μg/mL, respectively.

The mechanism by which the tannins are having an effect on delaying or inhibition of L3 exsheathment is not fully understood. The process of exsheathment is triggered by the presence of carbon dioxide and carbonic acid at 38°C. The sheath of the L3 is composed mainly of proline rich protein, with some lipids and carbohydrates (Bird and Rogers 1956). It is already established that the exsheathment process involves

the release of an exsheathment fluid which contains a mixture of enzymes including proteinases that act on the larval sheath proteins. Speculation has been made that tannins may be effecting the exsheathment process by inhibiting the enzymes present in the exsheathment fluid, or by forming complexes with the sheath proteins inhibiting the enzymes from accessing the protein. It has been shown in Chapter 8 that calliandra CT is a potent inhibitor of the digestive enzymes, trypsin and pancreatic α-amylase at very low CT concentrations. The inhibition of digestive enzymes has also been demonstrated by other workers (Gu et al. 2001; Kazeem et al. 2013; Horigome et al. 1988). It is possible then to speculate that the enzymes released during the exsheathment process may also be inhibited by calliandra CT and mimosa extract. The L3 sheath is the L2 cuticle that has separated to become a loose sheath over laying the new L3 cuticle. The L3 does not feed, therefore, the inhibition of larval exsheathment does not result from the ingestion of tannins.

9.6 Conclusion

It can be seen from the results that purified calliandra CT and mimosa extracts do have anthelmintic effects against the different stages of the life cycle of *H. contortus* and *T. colubriformis.*

Calliandra CT and mimosa extract both inhibited egg hatching. For callinadra CT there were differences in egg hatch between *H. contortus* susceptible and *H. contortus* MLs resistant strain and also between the *H. contortus* susceptible and *T. colubriformis* susceptible strain. The effects of mimosa extract were only significant for the *H. contortus* susceptible strain.

Feeding of the L1 was inhibited by both calliandra CT and mimosa extract but was not different between any of the worm strains for either tannin.

Calliandra CT was effective in reducing larval exsheathment, with differences between *H. contortus* susceptible and *H. contortus* MLs resistant strain and also between the *H. contortus* susceptible and *T. colubriformis* susceptible strain. There was no effects on inhibition of larval exsheathment due to mimosa extract.

Larval migration was inhibited by calliandra CT, however, there were no effects due to worm species. Mimosa had no effect on inhibition of larval migration.

CHAPTER 10 GENERAL DISCUSSION AND IMPLICATIONS OF RESEARCH

10.1 Introduction

Calliandra is an important shrub legume in the tropics due to its value as a high protein forage; however, it contains significant levels of CT. The potential nutritional value of this legume together with its possible anthelmintic properties (high tannin, 12 % DM) give calliandra added dimension as a feed.

Condensed tannins are capable of binding and/or precipitating water-soluble proteins (Bate-Smith 1973, Haslam 1989), and also forming complexes with minerals and carbohydrates, including cellulose. Condensed tannin can exist as free or bound to protein or fibre, with these fractions being interchangeable and pH dependent. Condensed tannins thus have the potential to bind digestive enzymes, Rubisco (F1 leaf protein) or both (Mole and Waterman 1987), inhibiting protein release in the gut and therefore restricting protein digestion and absorption of amino acids by the animal. Calliandra has a very good quality amino acid profile (Widiawati and Teleni 2004), however, a significant proportion of the N is excreted as faeces (Perez-Maldonado and Norton 1996, Widiawati 2002, Cresswell 2007). Furthermore, DMD has been shown to decrease when feeding a diet containing CT (Perez-Maldonado and Norton 1996, Cresswell 2007, Aufrère et al. 2013).

The protein-CT complex is possibly the most important CT complex with respect to nutritional interactions in the gut and anthelmintic effects. Two possible nutritional outcomes relating to protein-CT complexing and dissociation can occur. Firstly, the complexing of CT and protein in the rumen will result in enhanced by-pass protein if conditions, mainly pH are conducive to dietary protein remaining bound to CT during passage through the rumen, and thus protecting the dietary protein from microbial degradation. The only advantageous nutritional outcome of this by-pass protein is if the pH conditions post-rumen change and the protein-CT complex is dissociated allowing the protein to be digested and amino acid absorbed. Secondly, if post-rumen conditions in the gut are not conducive to releasing protein from the CT

complex; the protein will remain bound and be excreted in the faeces, losing a valuable source of dietary protein. An added complication is the inhibition of digestive enzymes due to the potential of CT to form complexes with enzymes in the small intestine.

Another novel approach reported extensively in the literature (Chapter 2) is the anthelmintic properties of plants containing high concentrations of CT. The CT is proposed to be the agent responsible for the anthelmintic properties. Reports of anthelmintic effects are varied from no effect to having significant impact on egg hatching, development and feeding of L1 and exsheathment and motility of L3, Male:female and worm burdens. The properties of these plants seem promising; however the mechanism of action still remains unclear.

A significant problem in global ruminant livestock industries is parasitism by GI nematodes. Parasitism causes production losses and has varying effects from not detectable to death. The use of commercial drenches, aside from resistance are very effective, however, they are becoming unsustainable due to resistance. The marketing of new commercial anthelmintics are infrequent and society is putting pressure on industries to provide chemical free foods and fibres. Over the past 10-15 years anthelmintic resistance has increased rapidly and is now even catastrophic in some regions. Resistance is evident worldwide, even with reports of resistance to Monepantal in New Zealand less than 4 years after its introduction (Love et al. 2003). Attempts are being undertaken to slow this down and strategies developed to try to overcome this, but unfortunately the worm is still winning.

10.2 Purpose of the Study

The main purpose of this study was to determine the dynamics of binding and dissociation of calliandra CT and to determine any *in vitro* effects of CT on digestive enzymes and different stages of the nematode life cycle. The *in vivo* (Chapter 5) experiment compared digesta from lambs fed high CT (calliandra) or no CT (lucerne) diets and in the presence and absence of either of the worm species *H. contortus* and *T. colubriformis* or no worms to determine if there were any interaction between worms, diet, digesta pH and CT fractions. These two worm

species are the most important production associated species in the tropics for small ruminants. This study also examined if the presence of these nematodes had an effect on concentration of CT fractions in different segments of the GI tract. *In vitro* experiments were also used to examine binding and dissociation of CT extracted from calliandra, with Rubisco and cellulose, at pH values from 1-9 and also at 0.5, 3, 8 and 24 h of complexing without the influence of digesta and GI secretions. Enzyme inhibition studies were conducted to determine if calliandra CT inhibited the activity of the digestive enzymes trypsin and pancreatic α-amylase. *In vitro* assays were also used to determine if purified calliandra CT and also a mimosa extract had any potential anthelmintic effects and an ability to disrupt the nematode life cycle. Comparisons were also made between each step in the methods of analyzing the three fractions of CT (free, protein and fibre-bound) using the two methods reported by (Terrill et al. 1992b) and (Perez-Maldonado 1994) to determine which of these methods were most suited to analyzing both plant material and lamb digesta. Condensed tannin was extracted and purified from calliandra for the internal standard in all CT assays, and also for use in the *in vitro* work (Chapters 7, 8 and 9). Rubisco was extracted and purified from Spinach for the complexing assays (Chapter 7), with attempts to extract Rubisco from calliandra unsuccessful.

10.3 pH and Condensed Tannin Complexing

The predominant findings of the work described in chapter 5 were that GI segment significantly influenced the overall mean concentration of all fractions of CT and total CT. The pH and free CT concentration and also between pH and total CT were well correlated. The interaction between CT and pH was also clearly demonstrated in chapter 7 with the *in vitro* study showing that relationships existed between pH and free CT and between pH and protein-bound CT. Free CT and protein-bound CT were also well correlated, confirming that these two fractions are in fact interchangeable.

Stable protein-CT complexes first appear in the rumen. The protein for this complex may come from a number of sources, Rubisco, rumen microbes or the L3. From Chapter 5 it can be seen that the greatest percent of protein-bound CT of all of the segments was found to exist in the rumen, therefore, the potential exists for all of these complexes to form. Although the CT dynamics clearly show in Chapter 5 that

the majority of CT is in the free form in both the abomasum and the duodenum, a higher portion was protein-bound in the ileum. Unfortunately, there was no jejunum sample to analyse but extrapolation from pH values would suggest that there would be significant amounts of CT bound to protein in this segment. There may be time in the duodenum and abomasum to start protein digestion but once the pH rose in the ileum and probably the jejunum protein and digestive enzymes could again complex with CT. Protein was not available for digestion and absorption as most was found to be excreted in the faeces as reported by Cresswell (2007), postulating that the CT was somehow inhibiting the proteolytic enzymes or rendering the enzymes inactive or actually still protein bound which was not fully accounted for in the CT assay.

10.4 Condensed Tannin and Digestive Enzyme Activity

The purpose of the enzyme inhibition studies in Chapter 8 was to investigate calliandra CT inhibition of the digestive enzymes trypsin and pancreatic α–amylase *in vitro*. The pH conditions in the *in vivo* work in Chapter 5 showed that significant amounts of protein-CT complexes were present in the rumen and post duodenum. The *in vitro* study in Chapter 7 also confirmed that at the pH of the rumen and post duodenum, protein-CT complexes were dominant. Results from Chapter 8 demonstrated that calliandra CT does inhibit the activity of the digestive enzymes, trypsin and pancreatic α-amylase. It is therefore, reasonable to suggest that this would be occurring in ruminants fed a diet high in calliandra CT and would contribute to increased faecal N output and the 15% reduction in DMD reported by Cresswell (2007). The relationship between the digestive enzymes and CT is well correlated. A highly significant, positive quadratic relationship was found to exist between percent inhibition of trypsin as well as pancreatic α–amylase and concentration of CT. Maximum inhibition of trypsin by CT was 100% at a concentration of 0.2 mg/mL; however, maximum inhibition of pancreatic α–amylase was only 96% at 0.025 mg/mL CT. A diet of 100% calliandra was fed to the lambs in Chapter 5, which contained 12 % DM CT. Digesta CT concentrations were estimated from the CT data in Chapter 5 and also from volume data of (Cresswell 2007). The CT concentration in the duodenum and the ileum ranged from 9-26 mg/mL which is far in excess of the CT concentrations of 0.2 mg/mL and 0.025 mg/mL that resulted in maximum inhibition of trypsin and pancreatic α–amylase, respectively. The

concentration of trypsin and pancreatic α–amylase, however, were not measured in the digesta samples collected.

10.5 Condensed Tannin and Anthelmintic Effects on Exogenous Life Stages of Nematodes

The main objective of the experiment described in Chapter 9 was to investigate anthelmintic effects on egg hatching, L1 feeding and L3 exsheathment and motility *in vitro*. Tannins have been shown to have effects on different stages of the nematode life cycle with the use of *in vitro* assays. Variable results have been reported and are dependent on the type, purity and concentration of tannin from the plant extract and there are also differences due to nematode species. Not all effects detected *in vitro* can be correlated with what happens *in vivo* due to the different physiological conditions present in the GI tract and the interactions with constituents of digesta and secretions. It was found that calliandra CT and mimosa extract are capable of disrupting different stages of the lifecycle *in vitro* of *H. contortus* susceptible, *H. contortus* MLs resistant and *T. colubriformis* susceptible strains.
Calliandra CT had an *in vitro* effect of inhibition of egg hatching, feeding of L1 and exsheathment and motility of L3. Mimosa extract, however, was found to only be effective at inhibiting egg hatching and L1 feeding. This study used concentrations of 200, 400, 800 and 1000 µg/mL of the 93.4% pure calliandra CT (Chapter 3) CT and mimosa extract from the bark of *Acacia mearnsii* (black wattle) which contains 68.3-72.3 % tannins. The different tannin compositions and purity of the mimosa extract and the calliandra CT, and the fact that the tannin content is lower in the mimosa extract may account for the differences in the anthelmintic effects. The difference in the effect may also be attributed to the type of proanthocyanidin that is predominant in the plant as well as the ratio of these.

10.6 Problems with Condensed Tannin Detection and Therefore Predictions of Condensed Tannin Affects

It is interesting to note that the pH range at which protein formed the most stable *in vitro* complex with CT was 3-9. The highest amount of protein bound to CT as a percentage of total CT detected was 99% at pH 4.5. This was, however, not always

the case *in vivo*. At pH 4.8 in the duodenum, most of the CT was in the free fraction (unbound) and only in the duodenum and lower GI tract were there significant percentages of protein-bound CT. This may suggest that the assay for protein-bound CT may have deficiencies due to changes in the CT, interaction with digesta or other GI tract secretions and be underestimating the amount of protein-bound CT. The results in Chapter 5 also clearly demonstrates that not all of the total CT that was ingested could be detected. This was the case for all segments above pH 4.8. This is also in agreement with the *in vitro* work that showed underestimation of CT at pH 4.5-9. Similar problems have also been reported by Terrill et al. (1992b) and Perez-Maldonado (1994). If this is the case, then it is more difficult to predict from the *in vivo* results which segments of GI tract CT is available to interact with Rubisco, nematode adults, larvae or eggs and so be exerting an anthelmintic effect. It appears that other factors are influencing binding and dissociation or interfering with the assay such as, GI secretions, digesta or ES products from the worm. It is possible that a change in chemical structure of the CT has occurred due to not all of CT able to be detected in the assay, and is influencing the detection of CT in the assay. Further work to investigate this inconsistency between protein-CT complexing *in vivo* and *in vitro* is warranted.

10.7 Influence of Worms on pH and Condensed tannin Concentration in the Gastrointestinal Tract

One of the objectives of the experiment described in Chapter 5 was to determine if worm or diet had any interaction on pH or CT concentrations along the GI tract. Worm infection affected pH, and this was found in the lower GI tract segments, ileum, caecum and colon. In the ileum, digesta from lambs infected with *H. contortus* which inhabits the abomasum, had the lowest pH compared with digesta from lambs infected with *T. colubriformis* or no infection. In the caecum pH was lowest in digesta of lambs infected with *T. colubriformis* compared with no infection but not different to that infected with *H. contortus* and in the colon. The pH was lowest in digesta of lambs infected with *T. colubriformis* compared with no infection and infected with *H. contortus*. It would be conceivable that *T. colubriformis* may inhabit these segments as they usually establish in the first three meters of the small intestine but due to immune response may be shifted downstream. Calliandra, the high CT

forage, increased pH in the abomasum and lower GI tract segments, ileum, caecum and colon compared with feed containing no CT.

10.8 Condensed Tannin Complexing With Time

One of the significant findings from Chapter 7 was that complexing time did not affect the concentration of free, protein-bound or fibre-bound CT in these *in vitro* assays. This is contrary to the findings of Jones and Mangan (1977) and Terrill et al. (1994). Further investigation is warranted to check if complexing at different times in the presence of liquid from digesta extracted from the rumen and duodenum of lambs, previously fed a non-tannin diet, changes the outcome.

10.9 Comparison of Assays for Condensed Tannin

The main objective of the experiment described in Chapter 4 was to determine if there was any difference in the results obtained when analysing plant material and digesta for free, protein-bound, fibre-bound CT and also calculated total CT, using the method of Perez-Maldonado (1994) and modified method of Terrill et al. (1992b). Calliandra and desmanthus were used for analysis of the plant material and digesta from lambs previously fed calliandra was used to make the digesta comparisons. If analysing for total CT concentration in plants, then either method could be used as the results were not significantly different. However, in analysing digesta the method of Perez-Maldonado (1994) proved to produce a significantly higher value for total CT than the modified method of Terrill et al. (1992b). The concentration of protein-bound CT was significantly higher in both plants and digesta analysed using the modified method of Terrill et al. (1992b) and the concentration of fibre-bound CT was significantly lower in both plants and digesta analysed using the modified method of Terrill et al. (1992b). For the desmanthus there was also a higher concentration of free CT when analysed using the method of Perez-Maldonado (1994). The results, however, may be more reliable for calliandra as the CT standards used for the analysis are species specific. There was no difference in concentration of total CT but the concentration of protein-bound CT was higher in digesta analysed by the modified method of Terrill et al. (1992b). This

method was much less complicated than the other but the downside is that it does use much higher concentrations of the highly toxic chemical mercaptoethanol.

10.10 Use of Internal Standards for the Analysis of Condensed Tannin

Both CT from calliandra and Rubisco from spinach were able to be extracted on a large scale and purified to be used in the *in vitro* work outlined in Chapter 7. The pure CT was also used as the internal standard as it is far more suitable to use the CT from the plant being assayed, as it will give an accurate tannin concentration of that particular plant instead of using tannic acid or other commercial tannin standards and expressing concentration for example as tannic acid equivalents. Not all CT have the same chemical and physical properties. Prodelphinidin and proanthocyanidin ratios may be different between different plants and the same standard may give inaccurate results. Using species specific CT as the internal standard is certainly the most appropriate option when anaylsing for CT and should be adopted by laboratories, although somewhat time consuming will give more accurate results.

10.11 Potential Anthelmintic Effects of Condensed Tannin

Chapters 5 and 7 clearly demonstrates that pH does affect the concentrations of free, protein-bound and fibre-bound CT and there is potential for interaction of CT with both species of nematodes along the GI tract. The *H. contortus* inhabits the abomasum and ingests and bathes in sheep blood so the negative effect that CT had on egg production (Cresswell 2007), must have been from contact with the cuticle or absorbed into circulation from ingested blood. Cresswell (2007) found CT staining in the pharynx, intestines and vulval flap of *H. contortus* and one female was found to have some staining on the surface cuticle striae. In two females faint staining was found in the edges of the oesophagus. In another study using *H. contortus* exposed to tannin-rich plants, structural changes were reported in the cuticle and buccal areas suggesting an effect on worm motility, reproduction and nutrition (Martinez-Ortiz-de-Montellano et al. 2013). It is possible that the CT may have an effect on the eggs, which are permeable in this nematode, as the eggs proceed down the lower GI tract where there the protein-bound CT percentages are high. Cresswell (2007) however,

found that worm burdens in second generation were not affected by egg exposure to CT in GI tract.

T. colubriformis is a duodenal grazer and would be able to ingest free, protein-bound and fibre-bound CT in the duodenum. Results from Chapter 5 indicate that this duodenal worm would mostly be exposed to CT in the unbound form, suggesting that very little CT would be complexing with the nematode cuticle or eggs. In contrast, the *in vitro* results from Chapter 7 would suggest that the CT is mostly in the protein-bound fraction, and hence able to complex with the nematode cuticle and eggs and this may be responsible for the decrease in egg production. The eggs have exposure along the GI tract, however, are not permeable in this species.

10.12 Conclusion and Implications of the Research

In conclusion calliandra contains a high concentration of CT and has potential as a high protein feed. There is evidence that feeding CT rich calliandra is exerting some anthelmintic effect on *H. contortus* and *T. colubriformis* (Cresswell 2007). It is clear that pH is a major influence on the proportion of CT in each of the three fractions of CT, and hence availability to interact with the worms, plant proteins, rumen microbes and digestive enzymes. It has been confirmed *in vivo* that protein is complexed in the rumen and also post duodenum, with *in vitro* studies confirming that trypsin is inhibited by CT. With this in mind it may help account for the increased loss of N in the faeces and decreased DMD in the lambs studied and anthelmintic effects reported by Cresswell (2007) with lambs fed a diet of 100% calliandra. It was also confirmed that calliandra CT and mimosa extract are capable of disrupting different stages of the lifecycle *in vitro* of *Hc.contortus* susceptible, *Hc.contortus* MLs resistant and *Tc.colubriformis* susceptible strains with Calliandra CT exerting a greater effect than the mimosa extract. It is clear that calliandra CT had an *in vitro* effect on effect of inhibition of egg hatching, feeding of L1 and exsheathment and motility of L3. mimosa extract was found to only be effective at inhibiting egg hatching and L1 feeding.

Future experiments to follow on from the results presented in this thesis include enzyme kinetic studies, further investigation into the method of CT detection in

digesta and *in vitro* complexing assays at different times in the presence of digesta. Repeating the *in vitro* LMIA at higher CT concentration and the LFIA at lower CT concentrations would also be useful, as well as conducting adult motility assays. It would be interesting to determine the type of enzyme inhibition, be it competitive or non-competitive with enzyme kinetic studies and to determine the dynamics of CT-enzyme complexing in the presence of Rubisco. There is enough evidence to suggest that other factors are interacting with the CT assay and producing false readings for each fraction of CT *in vivo*, therefore, more studies relating to detection of CT in digesta need to be conducted. Further investigation is also warranted to determine CT complexing at different times in the presence of liquid from digesta extracted from the rumen and duodenum of lambs, previously fed a non-tannin diet, to determine if this changes the dynamics of complexing in the assay, and is more in line with results from the *in vivo* complexing. Repeating the *in vitro* EHA, LFIA, LMIA and LEA with a greater number of replicates and a wider range of CT concentrations is also warranted.

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APPENDIX 1: STATISTICS TABLES

Chapter 4 Statistics Tables

Table A1.4.1 One-way ANOVA table for comparison of free, protein-bound, fibre-bound and total condensed tannin in desmanthus using the modified method of Terrill et al. (1992b) and the method of Perez-Maldonado (1994).

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
free	Between Groups	82.098	1	82.098	10.112	.013
	Within Groups	64.949	8	8.119		
	Total	147.047	9			
protein	Between Groups	32.856	1	32.856	3111.515	< .001
	Within Groups	.084	8	.011		
	Total	32.941	9			
fibre	Between Groups	.658	1	.658	56.905	< .001
	Within Groups	.092	8	.012		
	Total	.750	9			
total	Between Groups	17.137	1	17.137	2.124	.183
	Within Groups	64.561	8	8.070		
	Total	81.698	9			

Table A1.4.2 One-way ANOVA table for comparison of free, protein-bound, fibre-bound and total condensed tannin in calliandra using the modified method of Terrill et al. (1992b) and the method of Perez-Maldonado (1994).

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
free	Between Groups	1.348	1	1.348	.515	.493
	Within Groups	20.949	8	2.619		
	Total	22.297	9			
protein	Between Groups	4.361	1	4.361	176.569	< .001
	Within Groups	.198	8	.025		
	Total	4.558	9			
fibre	Between Groups	1.056	1	1.056	44.784	< .001
	Within Groups	.189	8	.024		
	Total	1.245	9			
total	Between Groups	.003	1	.003	.001	.973
	Within Groups	19.611	8	2.451		
	Total	19.614	9			

Table A1.4.3 One-way ANOVA table for comparison of free condensed tannin in calliandra using the modified method of Terrill et al. (1992b) and Perez-Maldonado (1994) with modification to compare extraction protocols of vortex versus mixing wheel.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
free	Between Groups	9.183	2	4.592	1.953	.172
	Within Groups	39.963	17	2.351		
	Total	49.146	19			

Table A1.4.5 Non-parametric Kruskal Wallis table for comparison of protein-bound condensed tannin in calliandra using the modified method of Terrill et al. (1992b) and Perez-Maldonado (1994) with modification to compare extraction protocols.

	protein
Chi-Square	12.909
df	2
Asymp. Sig.	.002

Table A1.4.6 MANOVA table for comparison of free, protein-bound, fibre-bound and total condensed tannin in calliandra using the modified method of Terrill et al. (1992b) and Perez-Maldonado (1994) with modification to compare extraction mixtures.

Extraction Mixtures: Univariate Tests

Dependent Variable		Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
free	Contrast	4.521	1	4.521	1.011	.322	.031
	Error	143.055	32	4.470			
protein	Contrast	.619	1	.619	30.444	< .001	.488
	Error	.650	32	.020			
fibre	Contrast	.158	1	.158	2.213	.147	.065
	Error	2.289	32	.072			
total	Contrast	3.021	1	3.021	.771	.387	.024
	Error	125.429	32	3.920			

The F tests the effect of sds. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Table A1.4.7 MANOVA table for comparison of free, protein-bound, fibre-bound and total condensed tannin in calliandra using the modified method of Terrill et al. (1992b) and Perez-Maldonado (1994) with modification to compare extraction times.

Extraction Times: Univariate Tests

Dependent Variable		Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
free	Contrast	20.634	3	6.878	1.539	.223	.126
	Error	143.055	32	4.470			
protein	Contrast	2.984	3	.995	48.940	< .001	.821
	Error	.650	32	.020			
fibre	Contrast	.728	3	.243	3.393	.030	.241
	Error	2.289	32	.072			
total	Contrast	19.852	3	6.617	1.688	.189	.137
	Error	125.429	32	3.920			

The F tests the effect of sdsxextrt. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Table A1.4.8 MANOVA table for comparison of free, protein-bound, fibre-bound and total condensed tannin in calliandra using the modified method of Terrill et al. (1992b) and Perez-Maldonado (1994) with modification to compare wash versus no wash in the fibre-bound analysis.

Wash versus no wash: Univariate Tests

Dependent Variable		Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
free	Contrast	25.268	7	3.610	.807	.587	.150
	Error	143.055	32	4.470			
protein	Contrast	3.487	7	.498	24.510	< .001	.843
	Error	.650	32	.020			
fibre	Contrast	13.006	7	1.858	25.972	< .001	.850
	Error	2.289	32	.072			
total	Contrast	40.584	7	5.798	1.479	.210	.244
	Error	125.429	32	3.920			

The F tests the effect of washing. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Table A1.4.9 Independent samples t-test table for comparison of free, protein-bound, fibre-bound and total condensed tannin in rumen digesta from sheep previously fed calliandra and using the modified method of Terrill et al. (1992b) for the analysis of free CT and methods of Terrill et al. (1992b) and Perez-Maldonado (1994).

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
free	Equal variances assumed	2.732	.142	.880	7	.408	.09434	.10725	-.15925	.34793
	Equal variances not assumed			.977	4.863	.375	.09434	.09654	-.15595	.34463
protein	Equal variances assumed	2.875	.134	-9.902	7	< .001	-.68110	.06879	-.84376	-.51844
	Equal variances not assumed			-11.066	4.601	< .001	-.68110	.06155	-.84353	-.51867
fibre	Equal variances assumed	5.840	.046	6.257	7	< .001	.92455	.14776	.57517	1.27394
	Equal variances not assumed			7.041	4.311	.002	.92455	.13130	.57014	1.27897
total	Equal variances assumed	1.458	.266	9.764	7	< .001	.33779	.03460	.25598	.41960
	Equal variances not assumed			10.034	6.991	< .001	.33779	.03367	.25816	.41742

Chapter 5 Statistics Tables

Table A1.5.1. MANOVA table for comparison of pH of digesta from rumen, abomasum, duodenum, jejunum, ileum, caecum and colon of lambs on either a diet of lucerne pellets or calliandra and infected with either of the worm species *T. colubriformis* (Tc) or *H. contortus* (Hc) or no worm infection (controls).

Tests of Between-Subjects Effects

Source	Dependent Variable	Type IV Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	rumen	.048[a]	5	.010	.827	.547
	abomasum	8.526[b]	5	1.705	6.294	.002
	duodenum	5.660[c]	5	1.132	1.907	.143
	jejunum	.430[d]	5	.086	1.089	.400
	ileum	.636[e]	5	.127	3.821	.016
	caecum	.679[f]	5	.136	11.455	.000
	colon	.407[g]	5	.081	8.478	.000
Intercept	rumen	1091.924	1	1091.924	93624.65	.000
	abomasum	295.894	1	295.894	1092.174	.000
	duodenum	685.859	1	685.859	1155.484	.000
	jejunum	944.351	1	944.351	11969.50	.000
	ileum	1285.274	1	1285.274	38615.39	.000
	caecum	1136.644	1	1136.644	95840.67	.000
	colon	1197.313	1	1197.313	124704.5	.000
diet	rumen	.002	1	.002	.182	.675
	abomasum	5.009	1	5.009	18.488	.000
	duodenum	2.127	1	2.127	3.583	.075
	jejunum	.148	1	.148	1.870	.188
	ileum	.284	1	.284	8.540	.009
	caecum	.496	1	.496	41.862	.000
	colon	.177	1	.177	18.450	.000
worm	rumen	.037	2	.018	1.585	.232
	abomasum	.563	2	.282	1.040	.374
	duodenum	2.307	2	1.154	1.943	.172
	jejunum	.019	2	.010	.121	.887
	ileum	.281	2	.141	4.223	.031
	caecum	.102	2	.051	4.304	.030
	colon	.152	2	.076	7.915	.003
diet * worm	rumen	.016	2	.008	.683	.518
	abomasum	2.737	2	1.368	5.050	.018
	duodenum	1.100	2	.550	.927	.414
	jejunum	.288	2	.144	1.824	.190
	ileum	.072	2	.036	1.078	.361
	caecum	.021	2	.010	.867	.437
	colon	.025	2	.012	1.287	.300
Error	rumen	.210	18	.012		
	abomasum	4.877	18	.271		
	duodenum	10.684	18	.594		
	jejunum	1.420	18	.079		
	ileum	.599	18	.033		
	caecum	.213	18	.012		
	colon	.173	18	.010		
Total	rumen	1115.192	24			
	abomasum	307.963	24			
	duodenum	718.454	24			
	jejunum	961.352	24			
	ileum	1313.848	24			
	caecum	1157.930	24			
	colon	1221.379	24			
Corrected Total	rumen	.258	23			
	abomasum	13.403	23			
	duodenum	16.344	23			
	jejunum	1.850	23			
	ileum	1.235	23			
	caecum	.893	23			
	colon	.580	23			

a. R Squared = .187 (Adjusted R Squared = -.039)
b. R Squared = .636 (Adjusted R Squared = .535)
c. R Squared = .346 (Adjusted R Squared = .165)
d. R Squared = .232 (Adjusted R Squared = .019)
e. R Squared = .515 (Adjusted R Squared = .380)
f. R Squared = .761 (Adjusted R Squared = .694)
g. R Squared = .702 (Adjusted R Squared = .619)

Table A1.5.2. Nonparametric Kruskal Wallis table for comparison of the overall means for pH of digesta from rumen, abomasum, duodenum, jejunum, ileum, caecum and colon of lambs pooled for diet and worm species.

Test Statistics[a,b]

	pH
Chi-Square	222.481
df	6
Asymp. Sig.	< .001

a. Kruskal Wallis Test
b. Grouping Variable: segment

Table A1.5.3. Nonparametric Kruskal Wallis table for comparison of the means for concentration of free, protein-bound, fibre-bound and total condensed tannin in digesta from rumen (R), abomasum (A), duodenum (D), ileum (I), caecum (CM) and colon (CN) of lambs fed a diet of calliandra and infected with either of the worm species *T. colubriformis, H. contortus* or no worm infection (controls).

Test Statistics[a,b]

	RFree	RProtein	RFibre	RTotal	AFree	AProtein	AFibre	ATotal
Chi-Square	3.731	.154	1.192	1.077	1.192	1.852	4.192	.462
df	2	2	2	2	2	2	2	2
Asymp. Sig.	.155	.926	.551	.584	.551	.396	.123	.794

	DFree	DProtein	DFibre	DTotal	IFree	IProtein	IFibre	ITotal
Chi-Square	3.860	1.114	.038	3.231	4.815	1.654	.731	.500
df	2	2	2	2	2	2	2	2
Asymp. Sig.	.145	.573	.981	.199	.090	.437	.694	.779

	CMFree	CMProtein	CMFibre	CMTotal
Chi-Square	5.077	3.929	.500	.857
df	2	2	2	2
Asymp. Sig.	.079	.140	.779	.651

	CNFree	CNProtein	CNFibre	CNTotal
Chi-Square	3.281	6.000	2.125	.167
df	2	2	2	2
Asymp. Sig.	.194	.050	.346	.920

a. Kruskal Wallis Test
b. Grouping Variable: **Worm**

Table A1.5.4. Non-parametric Kruskal Wallis table for comparison of the means of data pooled for worms for concentration of free, protein-bound, fibre-bound and total condensed tannin as % dry matter in feed and digesta from rumen, abomasum, duodenum, ileum, caecum and colon of lambs fed a diet of calliandra.

Test Statistics[a,b]

	Free	Protein	Fibre	Total
Chi-Square	35.033	45.281	53.518	40.910
df	5	5	5	5
Asymp. Sig.	< .001	< .001	< .001	< .001

a. Kruskal Wallis Test
b. Grouping Variable: **segment**

Table A.5.5 Linear mixed Model table for relationships between (A) Protein-bound CT and free CT; (B) Fibre-bound CT and free CT; (C) Total CT and free CT; (D) free CT and protein-bound CT; (E) fibre bound CT and protein bound CT; (F) total CT and protein bound CT; (G) free CT and fibre bound CT; (H) protein bound CT and fibre bound CT; (I) total CT and fibre bound CT; (J) free CT and total CT; (K) protein bound CT and total CT; (L) fibre bound CT and total CT.

(A)

Estimates of Fixed Effects[a]

Parameter	Estimate	Std. Error	df	t	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Intercept	-1.083447	.938866	40.862	-1.154	.255	-2.979720	.812825
[Worm=1.00]	1.160536	.712381	8.523	1.629	.140	-.464824	2.785896
[Worm=2.00]	1.445790	.711336	8.481	2.032	.075	-.178495	3.070075
[Worm=3.00]	0[b]	0	.	.	.	.	.
[segment=1.00]	.449927	.529494	45.831	.850	.400	-.615995	1.515848
[segment=2.00]	11.149792	.802844	51.394	13.888	< .001	9.538315	12.761268
[segment=3.00]	1.832822	.805186	51.415	2.276	.027	.216660	3.448984
[segment=4.00]	.449108	.555245	46.673	.809	.423	-.668108	1.566324
[segment=5.00]	.078272	.592801	45.141	.132	.896	-1.115588	1.272132
[segment=6.00]	0[b]	0	.	.	.	.	.
Protein	.467313	.280978	53.850	1.663	.102	-.096049	1.030675

a. Dependent Variable: Free.

b. This parameter is set to zero because it is redundant.

(B)

Estimates of Fixed Effects[a]

Parameter	Estimate	Std. Error	df	t	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Intercept	2.217594	1.557679	53.999	1.424	.160	-.905365	5.340553
[Worm=1.00]	1.011126	.786727	8.312	1.285	.233	-.791276	2.813528
[Worm=2.00]	1.414474	.787490	8.338	1.796	.109	-.388760	3.217707
[Worm=3.00]	0[b]	0	.	.	.	.	.
[segment=1.00]	-1.007448	1.118006	47.984	-.901	.372	-3.255369	1.240473
[segment=2.00]	8.232333	1.372326	48.116	5.999	< .001	5.473257	10.991408
[segment=3.00]	-.528907	1.033432	47.909	-.512	.611	-2.606863	1.549049
[segment=4.00]	-.095598	.546423	45.852	-.175	.862	-1.195586	1.004390
[segment=5.00]	.035332	.589079	44.748	.060	.952	-1.151318	1.221983
[segment=6.00]	0[b]	0	.	.	.	.	.
Fibre	-.667012	.448763	48.119	-1.486	.144	-1.569253	.235228

a. Dependent Variable: Free.

b. This parameter is set to zero because it is redundant.

(C)

Estimates of Fixed Effects[a]

Parameter	Estimate	Std. Error	df	t	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Intercept	-2.601632	.448980	40.484	-5.795	< .001	-3.508717	-1.694547
[Worm=1.00]	.438811	.175243	6.079	2.504	.046	.011362	.866261
[Worm=2.00]	.471698	.178745	6.287	2.639	.037	.039123	.904273
[Worm=3.00]	0[b]	0	.	.	.	.	.
[segment=1.00]	1.467715	.261352	48.958	5.616	< .001	.942497	1.992933
[segment=2.00]	5.932923	.366872	51.924	16.172	< .001	5.196714	6.669131
[segment=3.00]	2.678504	.306998	52.986	8.725	< .001	2.062740	3.294268
[segment=4.00]	.700602	.252296	46.558	2.777	.008	.192921	1.208283
[segment=5.00]	.049839	.283792	43.795	.176	.861	-.522183	.621860
[segment=6.00]	0[b]	0	.	.	.	.	.
Total	.309051	.100588	47.172	3.072	.004	.106714	.511388
Total2	.027468	.006142	52.752	4.472	< .001	.015147	.039788

a. Dependent Variable: Free.

b. This parameter is set to zero because it is redundant.

(D)

Estimates of Fixed Effects[a]

Parameter	Estimate	Std. Error	df	t	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Intercept	2.501457	.250943	21.727	9.968	< .001	1.980655	3.022259
[Worm=1.00]	-.446785	.274658	8.076	-1.627	.142	-1.079116	.185546
[Worm=2.00]	-.507235	.283470	8.740	-1.789	.108	-1.151407	.136938
[Worm=3.00]	0[b]	0	.	.	.	.	.
[segment=1.00]	-.161660	.260338	45.795	-.621	.538	-.685756	.362436
[segment=2.00]	-3.909264	.711321	51.116	-5.496	< .001	-5.337221	-2.481307
[segment=3.00]	-2.473688	.267795	46.515	-9.237	< .001	-3.012571	-1.934805
[segment=4.00]	-.751350	.252673	45.163	-2.974	.005	-1.260207	-.242492
[segment=5.00]	.098825	.277352	43.916	.356	.723	-.460172	.657822
[segment=6.00]	0[b]	0	.	.	.	.	.
Free	.336801	.111827	50.397	3.012	.004	.112233	.561369
Free2	-.013921	.005728	52.289	-2.431	.019	-.025413	-.002430

a. Dependent Variable: Protein.

b. This parameter is set to zero because it is redundant.

(E)

Estimates of Fixed Effects[a]

Parameter	Estimate	Std. Error	df	t	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Intercept	1.528775	.735787	53.885	2.078	.043	.053539	3.004010
[Worm=1.00]	-.247801	.323441	8.132	-.766	.465	-.991555	.495953
[Worm=2.00]	-.256247	.323840	8.160	-.791	.451	-1.000487	.487994
[Worm=3.00]	0[b]	0	.	.	.	.	.
[segment=1.00]	.782071	.537658	49.102	1.455	.152	-.298337	1.862479
[segment=2.00]	-1.250888	.659761	49.277	-1.896	.064	-2.576539	.074762
[segment=3.00]	-1.529673	.497072	49.001	-3.077	.003	-2.528576	-.530769
[segment=4.00]	-.489065	.264005	46.229	-1.852	.070	-1.020410	.042279
[segment=5.00]	.187457	.285257	44.745	.657	.514	-.387170	.762084
[segment=6.00]	0[b]	0	.	.	.	.	.
Fibre	.318360	.215743	49.298	1.476	.146	-.115125	.751845

a. Dependent Variable: Protein.

b. This parameter is set to zero because it is redundant.

(F)

Estimates of Fixed Effects[a]

Parameter	Estimate	Std. Error	df	t	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Intercept	.243949	.388416	47.047	.628	.533	-.537422	1.025320
[Worm=1.00]	-.398761	.232446	8.256	-1.716	.123	-.931904	.134382
[Worm=2.00]	-.517986	.234818	8.496	-2.206	.057	-1.054024	.018053
[Worm=3.00]	0[b]	0	.	.	.	.	.
[segment=1.00]	.564178	.195066	47.620	2.892	.006	.171890	.956466
[segment=2.00]	-3.113022	.280280	49.778	-11.107	< .001	-3.676042	-2.550002
[segment=3.00]	-1.049392	.238813	50.991	-4.394	< .001	-1.528830	-.569954
[segment=4.00]	-.424374	.185503	45.912	-2.288	.027	-.797792	-.050956
[segment=5.00]	.115776	.205547	44.141	.563	.576	-.298440	.529992
[segment=6.00]	0[b]	0	.	.	.	.	.
Total	.504601	.081177	52.138	6.216	< .001	.341717	.667484
Total2	-.017652	.004756	49.513	-3.712	.001	-.027206	-.008097

a. Dependent Variable: Protein.

b. This parameter is set to zero because it is redundant.

(G)

Estimates of Fixed Effects[a]

Parameter	Estimate	Std. Error	df	t	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Intercept	3.134522	.144535	31.581	21.687	< .001	2.839961	3.429084
[Worm=1.00]	-.043881	.136433	7.179	-.322	.757	-.364874	.277111
[Worm=2.00]	.111133	.139436	7.456	.797	.450	-.214538	.436804
[Worm=3.00]	0[b]	0	.	.	.	.	.
[segment=1.00]	-2.170094	.166534	45.006	-13.031	< .001	-2.505510	-1.834678
[segment=2.00]	-2.597909	.413705	51.119	-6.280	< .001	-3.428408	-1.767409
[segment=3.00]	-1.947566	.168863	44.929	-11.533	< .001	-2.287689	-1.607443
[segment=4.00]	-.329723	.165131	45.422	-1.997	.052	-.662227	.002782
[segment=5.00]	-.168872	.185194	44.618	-.912	.367	-.541960	.204217
[segment=6.00]	0[b]	0	.	.	.	.	.
Free	-.020443	.036644	41.889	-.558	.580	-.094399	.053513

a. Dependent Variable: Fibre.

b. This parameter is set to zero because it is redundant.

(H)

Estimates of Fixed Effects[a]

Parameter	Estimate	Std. Error	df	t	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Intercept	2.896045	.243668	44.929	11.885	< .001	2.405251	3.386840
[Worm=1.00]	-.039944	.129420	9.025	-.309	.765	-.332591	.252703
[Worm=2.00]	.105913	.128990	8.945	.821	.433	-.186154	.397980
[Worm=3.00]	0[b]	0	.	.	.	.	.
[segment=1.00]	-2.189846	.163339	48.245	-13.407	< .001	-2.518217	-1.861474
[segment=2.00]	-2.604954	.234782	53.989	-11.095	< .001	-3.075666	-2.134243
[segment=3.00]	-1.762993	.235403	53.991	-7.489	< .001	-2.234950	-1.291037
[segment=4.00]	-.278618	.170141	49.375	-1.638	.108	-.620464	.063228
[segment=5.00]	-.182085	.184074	46.800	-.989	.328	-.552435	.188265
[segment=6.00]	0[b]	0	.	.	.	.	.
Protein	.094141	.077997	48.251	1.207	.233	-.062661	.250944

a. Dependent Variable: Fibre.

b. This parameter is set to zero because it is redundant.

(I)

Estimates of Fixed Effects[a]

Parameter	Estimate	Std. Error	df	t	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Intercept	2.485859	.288269	43.823	8.623	< .001	1.904826	3.066892
[Worm=1.00]	-.088878	.116648	7.950	-.762	.468	-.358162	.180406
[Worm=2.00]	.022482	.118877	8.214	.189	.855	-.250412	.295376
[Worm=3.00]	0[b]	0	.	.	.	.	.
[segment=1.00]	-2.060559	.165551	49.826	-12.447	< .001	-2.393106	-1.728011
[segment=2.00]	-2.959023	.232930	52.119	-12.703	< .001	-3.426406	-2.491641
[segment=3.00]	-1.671459	.195311	52.968	-8.558	< .001	-2.063209	-1.279709
[segment=4.00]	-.296909	.159594	47.910	-1.860	.069	-.617811	.023993
[segment=5.00]	-.166532	.179265	45.652	-.929	.358	-.527449	.194384
[segment=6.00]	0[b]	0	.	.	.	.	.
Total	.154445	.064355	49.684	2.400	.020	.025163	.283726
Total2	-.006906	.003912	52.968	-1.765	.083	-.014752	.000941

a. Dependent Variable: Fibre.

b. This parameter is set to zero because it is redundant.

(J)

Estimates of Fixed Effects[a]

Parameter	Estimate	Std. Error	df	t	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Intercept	5.560576	.282297	53.000	19.698	< .001	4.994360	6.126793
[Worm=1.00]	-.507497	.241506	53.000	-2.101	.040	-.991898	-.023096
[Worm=2.00]	-.473644	.253638	53.000	-1.867	.067	-.982378	.035090
[Worm=3.00]	0[b]	0	.	.	.	.	.
[segment=1.00]	-2.376210	.358092	53.000	-6.636	< .001	-3.094452	-1.657967
[segment=2.00]	-6.805674	.867329	53.000	-7.847	< .001	-8.545316	-5.066032
[segment=3.00]	-4.486650	.366662	53.000	-12.236	< .001	-5.222081	-3.751219
[segment=4.00]	-1.094262	.349091	53.000	-3.135	.003	-1.794451	-.394073
[segment=5.00]	-.089438	.386656	53.000	-.231	.818	-.864971	.686095
[segment=6.00]	0[b]	0	.	.	.	.	.
Free	1.441288	.134487	53.000	10.717	< .001	1.171540	1.711036
Free2	-.021555	.007402	53.000	-2.912	.005	-.036400	-.006709

a. Dependent Variable: Total.

b. This parameter is set to zero because it is redundant.

(K)

Estimates of Fixed Effects[a]

Parameter	Estimate	Std. Error	df	t	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Intercept	1.731386	.952458	37.879	1.818	.077	-.196967	3.659738
[Worm=1.00]	1.167635	.791301	8.764	1.476	.175	-.629770	2.965041
[Worm=2.00]	1.565345	.790400	8.730	1.980	.080	-.231143	3.361833
[Worm=3.00]	0[b]	0	.	.	.	.	.
[segment=1.00]	-1.715154	.508132	45.743	-3.375	.002	-2.738127	-.692181
[segment=2.00]	8.602635	.777796	50.363	11.060	< .001	7.040665	10.164604
[segment=3.00]	.124645	.780100	50.381	.160	.874	-1.441938	1.691228
[segment=4.00]	.204501	.533518	46.426	.383	.703	-.869149	1.278150
[segment=5.00]	-.119869	.568330	45.224	-.211	.834	-1.264389	1.024651
[segment=6.00]	0[b]	0	.	.	.	.	.
Protein	1.576247	.274439	53.005	5.744	< .001	1.025794	2.126700

a. Dependent Variable: Total.

b. This parameter is set to zero because it is redundant.

(L)

Estimates of Fixed Effects[a]

Parameter	Estimate	Std. Error	df	t	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Intercept	3.406347	1.899255	53.976	1.794	.078	-.401468	7.214162
[Worm=1.00]	.779524	.917710	8.275	.849	.420	-1.324554	2.883602
[Worm=2.00]	1.128871	.918674	8.301	1.229	.253	-.976294	3.234037
[Worm=3.00]	0[b]	0	.	.	.	.	.
[segment=1.00]	.043510	1.371784	48.323	.032	.975	-2.714172	2.801191
[segment=2.00]	7.299596	1.683686	48.467	4.335	< .001	3.915160	10.684032
[segment=3.00]	-1.809813	1.268075	48.239	-1.427	.160	-4.359122	.739495
[segment=4.00]	-.462122	.671352	45.976	-.688	.495	-1.813502	.889258
[segment=5.00]	.210052	.724233	44.762	.290	.773	-1.248842	1.668946
[segment=6.00]	0[b]	0	.	.	.	.	.
Fibre	.730120	.550579	48.475	1.326	.191	-.376613	1.836852

a. Dependent Variable: Total.

b. This parameter is set to zero because it is redundant.

Chapter 7 Statistics Tables

Table A1.7.1. Non-parametric Kruskall Wallis table for comparison of the means of the concentration of the fractions of condensed tannin after 0.5, 3, 8 or 24 h of complexing.

Test Statistics[a,b]

	CT
Chi-Square	.538
df	3
Asymp. Sig.	.911

a. Kruskal Wallis Test

b. Grouping Variable: Complex_Time

Table A1.7.2. Non-parametric Kruskall Wallis table for comparison of the means of the concentration of the fractions of condensed tannin, at pH 1-9 after 0.5, 3, 8 or 24 h of complexing.

Test Statistics[a,b]

	pH_1	pH_1.5	pH_2	pH_2.5	pH_3	pH_4	pH_4.5	pH_5	pH_6	pH_7	pH_8	pH_9
Chi-Square	1.581	.178	2.088	.050	.262	1.304	1.221	1.538	3.795	.814	1.745	.855
df	3	3	3	3	3	3	3	3	3	3	3	3
Asymp. Sig.	.664	.981	.554	.997	.967	.728	.748	.674	.284	.846	.627	.836

a. Kruskal Wallis Test

b. Grouping Variable: Complex_time

Table A1.7.3. Non-parametric Kruskall Wallis table for comparison of the means of concentration of the three fractions, free, protein-bound and fibre-bound condensed tannin (CT).

Test Statistics[a,b]

	CT
Chi-Square	259.772
df	2
Asymp. Sig.	< .001

a. Kruskal Wallis Test

b. Grouping Variable: Fraction

Table A1.7.4. Non-parametric Kruskal Wallis table for comparsion of the means of concentration of free, protein-bound and fibre-bound condensed tannin (mg) at pH 1-9.

Test Statistics[a,b]

	pH_1	pH_1.5	pH_2	pH_2.5	pH_3	pH_4	pH_4.5	pH_5	pH_6	pH_7	pH_8	pH_9
Chi-Square	8.000	10.455	10.203	10.203	8.375	8.346	10.240	7.731	7.385	9.881	9.846	8.858
df	2	2	2	2	2	2	2	2	2	2	2	2
Asymp. Sig.	.018	.005	.006	.006	.015	.015	.006	.021	.025	.007	.007	.012

a. Kruskal Wallis Test

b. Grouping Variable: Fraction

Table A1.7.5. Non-parametric Kruskal Wallis table for comparsion of the means of the concentration of total condensed tannin (CT) detected for pH 1-9.

Test Statistics[a,b]

	CT
Chi-Square	67.788
df	11
Asymp. Sig.	< .001

a. Kruskal Wallis Test

b. Grouping Variable: pH

Table A1.7.6. Non-parametric Kruskal Wallis table for comparison of means of condensed tannin (CT) after spiking buffer at pH 1-9 and water with 2 mg of CT.

Test Statistics[a,b]

	CT
Chi-Square	19.870
df	12
Asymp. Sig.	.070

a. Kruskal Wallis Test

b. Grouping Variable: pH

Chapter 8 Statistics Tables

Table A1.8.1 Spearman's correlation table for relationship between Trypsin inhibition and calliandra condensed tannin concentration.

Correlations

			CT_concentration	Trypsin_Inhibition
Spearman's rho	CT_concentration	Correlation Coefficient	1.000	.983**
		Sig. (2-tailed)	.	.000
		N	27	27
	Trypsin_Inhibition	Correlation Coefficient	.983**	1.000
		Sig. (2-tailed)	< .001	.
		N	27	27

**. Correlation is significant at the 0.01 level (2-tailed).

Table A1.8.2 Spearman's correlation table for relationship between pancreatic α–amylase inhibition and calliandra condensed tannin concentration.

Correlations

			Amylase_inhibition	CT_concentration
Spearman's rho	Amylase_inhibition	Correlation Coefficient	1.000	.984**
		Sig. (2-tailed)	.	.000
		N	21	21
	CT_concentration	Correlation Coefficient	.984**	1.000
		Sig. (2-tailed)	< .001	.
		N	21	21

**. Correlation is significant at the 0.01 level (2-tailed).

Chapter 9 Statistics Tables

Table A1.9.1 General linear model univariate analysis table for comparisons between calliandra condensed tannin concentration and worm strain (*H. contortus* susceptible, *H. contortus* resistant and *T. colubriformis* susceptible strain) for percent egg hatched for the egg hatch inhibition assay.

Tests of Between-Subjects Effects

Dependent Variable: percentegghatch

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	7957.065[a]	14	568.362	22.421	< .001
Intercept	273975.477	1	273975.477	10807.877	< .001
strain	4114.734	2	2057.367	81.160	< .001
CTconc	2975.995	4	743.999	29.350	< .001
strain * CTconc	1028.423	8	128.553	5.071	.001
Error	735.139	29	25.350		
Total	282632.279	44			
Corrected Total	8692.204	43			

a. R Squared = .915 (Adjusted R Squared = .875)

Table A1.9.2 ANOVA table for comparisons for calliandra condensed tannin concentration between/within worm strain (*H. contortus* susceptible, *H. contortus* resistant and *T. colubriformis* susceptible strain) for percent egg hatched for the egg hatch inhibition assay.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Hs	Between Groups	1261.313	4	315.328	7.968	.004
	Within Groups	395.722	10	39.572		
	Total	1657.035	14			
Ts	Between Groups	1368.239	4	342.060	18.612	< .001
	Within Groups	183.786	10	18.379		
	Total	1552.025	14			
Hr	Between Groups	1349.819	4	337.455	19.515	< .001
	Within Groups	155.631	9	17.292		
	Total	1505.450	13			

Table A1.9.3 General linear model univariate analysis table for comparisons between mimosa extract concentration and worm strain (*H. contortus* susceptible, *H. contortus* resistant and *T. colubriformis* susceptible strain) for percent egg hatched for the egg hatch inhibition assay.

Tests of Between-Subjects Effects

Dependent Variable: percentegghatch

Source	Type IV Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	8466.796[a]	14	604.771	22.461	< .001	.913
Intercept	298690.056	1	298690.056	11093.476	< .001	.997
Strain	6590.771	2	3295.385	122.392	< .001	.891
CTconc	725.880	4	181.470	6.740	.001	.473
Strain * CTconc	1150.145	8	143.768	5.340	< .001	.587
Error	807.745	30	26.925			
Total	307964.597	45				
Corrected Total	9274.541	44				

a. R Squared = .913 (Adjusted R Squared = .872)

Table A1.9.4 ANOVA table for comparison of percent egg hatch between 0 tannin, DMSO and Thiabendazole for the egg hatch assay.

Tests of Between-Subjects Effects

Dependent Variable: egghatch

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[b]
Corrected Model	30995.844[a]	8	3874.481	197.535	< .001	.989	1580.280	1.000
Intercept	98365.095	1	98365.095	5015.007	< .001	.996	5015.007	1.000
TEST	29312.336	2	14656.168	747.224	< .001	.988	1494.449	1.000
Strain	833.631	2	416.815	21.251	< .001	.702	42.502	1.000
TEST * Strain	849.877	4	212.469	10.832	< .001	.707	43.330	.999
Error	353.055	18	19.614					
Total	129713.993	27						
Corrected Total	31348.899	26						

a. R Squared = .989 (Adjusted R Squared = .984)

b. Computed using alpha = .05

Table A1.9.5 Non-parametric Kruskal Wallis table for comparison of percent feeding inhibition and worm strain (*H. contortus* susceptible, *H. contortus* resistant and *T. colubriformis* susceptible strain) for calliandra condensed tannin the larval feeding inhibition assay.

Test Statistics[a,b]

	percentfeedinginhibition
Chi-Square	.033
df	2
Asymp. Sig.	.984

a. Kruskal Wallis Test
b. Grouping Variable: Strain

Table A1.9.6 Non-parametric Kruskal Wallis table for comparison of percent feeding inhibition and calliandra condensed tannin concentration for the larval feeding inhibition assay.

Test Statistics[a,b]

	percentfeedinginhibition
Chi-Square	43.287
df	4
Asymp. Sig.	< .001

a. Kruskal Wallis Test
b. Grouping Variable: CTconc

Table A1.9.7 Non-parametric Kruskal Wallis table for comparison of percent feeding inhibition and calliandra condensed tannin concentration for (A) *H. contortus* susceptible, (B) *H. contortus* resistant and (C) *T. colubriformis* susceptible strain for the larval feeding inhibition assay.

(A)

Test Statistics[a,b]

	Hs
Chi-Square	13.796
df	4
Asymp. Sig.	.008

a. Kruskal Wallis Test
b. Grouping Variable: CTconc

(B)

Test Statistics[a,b]

	Hr
Chi-Square	13.796
df	4
Asymp. Sig.	.008

a. Kruskal Wallis Test
b. Grouping Variable: CTconc

(C)

Test Statistics[a,b]

	Ts
Chi-Square	13.796
df	4
Asymp. Sig.	.008

a. Kruskal Wallis Test
b. Grouping Variable: CTconc

Table A1.9.8 Non-parametric Kruskal Wallis table for comparison of percent feeding inhibition for pooled data for worm strain and calliandra condensed tannin concentration for the larval feeding inhibition assay.

Test Statistics[a,b]

	Inhibition
Chi-Square	43.287
df	4
Asymp. Sig.	< .001

a. Kruskal Wallis Test
b. Grouping Variable: CT

Table A1.9.9 Non-parametric Kruskal Wallis table for comparison of percent feeding inhibition and worm strain (*H. contortus* susceptible, *H. contortus* resistant and *T. colubriformis* susceptible strain) for mimosa extract for the larval feeding inhibition assay.

Test Statistics[a,b]

	percentfeedinginhibition
Chi-Square	.033
df	2
Asymp. Sig.	.984

a. Kruskal Wallis Test
b. Grouping Variable: Strain

Table A1.9.10 Non-parametric Kruskal Wallis table for comparison of percent feeding inhibition and mimosa extract concentration for the larval feeding inhibition assay.

Test Statistics[a,b]

	percentfeedinginhibition
Chi-Square	43.287
df	4
Asymp. Sig.	< .001

a. Kruskal Wallis Test
b. Grouping Variable: CTconc

Table A1.9.11 Non-parametric Kruskal Wallis table for comparison of percent feeding inhibition and mimosa extract concentration for (A) *H. contortus* susceptible, (B) *H. contortus* resistant and (C) *T. colubriformis* susceptible strain for the larval feeding inhibition assay.

(A)

Test Statistics[a,b]

	Hs
Chi-Square	13.796
df	4
Asymp. Sig.	.008

a. Kruskal Wallis Test
b. Grouping Variable: CT

(B)

Test Statistics[a,b]

	Hr
Chi-Square	13.796
df	4
Asymp. Sig.	.008

a. Kruskal Wallis Test
b. Grouping Variable: CT

(C)

Test Statistics[a,b]

	Ts
Chi-Square	12.923
df	4
Asymp. Sig.	.012

a. Kruskal Wallis Test
b. Grouping Variable: CT

Table A1.9.12 Non-parametric Kruskal Wallis table for comparison of percent feeding inhibition for pooled data for worm strain and mimosa extract concentration for the larval feeding inhibition assay.

Test Statistics[a,b]

	inhibition
Chi-Square	42.437
df	4
Asymp. Sig.	< .001

a. Kruskal Wallis Test
b. Grouping Variable: CT

Table A1.9.13 Non-parametric Kruskal Wallis table for overall comparison of percent larval feeding inhibition between 0 tannin, DMSO and Thiabendazole for the larval feeding inhibition assay.

Test Statistics[a,b]

	feedinginhibition
Chi-Square	17.885
df	3
Asymp. Sig.	< .001

a. Kruskal Wallis Test
b. Grouping Variable: TEST

1=0 tannin, 2=dmso,3=Ivermectin,4=TBZ

Table A1.9.14 Non-parametric Kruskal Wallis table for comparison of percent larval feeding inhibition and worm strain between 0 tannin, DMSO and Thiabendazole for the larval feeding inhibition assay.

Test Statistics[a,b]

	feedinginhibition
Chi-Square	1.361
df	2
Asymp. Sig.	.506

a. Kruskal Wallis Test
b. Grouping Variable: Strain

Table A1.9.15 Non-parametric Kruskal Wallis table for comparison of percent larval migration inhibition and worm strain (*H. contortus* susceptible, *H. contortus* resistant and *T. colubriformis* susceptible strain) for calliandra condensed tannin for the larval migration inhibition assay.

Test Statistics[a,b]

	percentmigration inhibition
Chi-Square	1.612
df	2
Asymp. Sig.	.447

a. Kruskal Wallis Test
b. Grouping Variable: Strain

Table A1.9.16 Non-parametric Kruskal Wallis table for comparison of percent larval migration inhibition and condensed tannin concentration for calliandra condensed tannin for the larval migration inhibition assay.

Test Statistics[a,b]

	percentmigration inhibition
Chi-Square	13.207
df	4
Asymp. Sig.	.010

a. Kruskal Wallis Test
b. Grouping Variable: CTconc

Table A1.9.17 Spearman's correlation table for relationship between calliandra condensed tannin concentration and percent larval migration inhibition.

Correlations

			percentmigrationinhibition	CTconc
Spearman's rho	percentmigrationinhibition	Correlation Coefficient	1.000	.348*
		Sig. (2-tailed)	.	.019
		N	45	45
	CTconc	Correlation Coefficient	.348*	1.000
		Sig. (2-tailed)	.019	.
		N	45	45

*. Correlation is significant at the 0.05 level (2-tailed).

Table A1.9.18 Non-parametric Kruskal Wallis table for comparison of percent larval migration inhibition pooled for worm strain between 0 tannin, DMSO, Levamisole and Ivermectin for the larval migration inhibition assay.

Test Statistics[a,b]

	percentmigration inhibition
Chi-Square	9.329
df	3
Asymp. Sig.	.025

a. Kruskal Wallis Test
b. Grouping Variable: TEST
1=0CT, 2=DMSO, 3= ivermectin, 4=Levamisole

Table A1.9.19 Non-parametric Kruskal Wallis table for comparison of percent larval migration inhibition and mimosa extract concentration for mimosa extract for the larval migration inhibition assay.

Test Statistics[a,b]

	percentmigration inhibition
Chi-Square	5.785
df	4
Asymp. Sig.	.216

a. Kruskal Wallis Test
b. Grouping Variable: CTconc

Table A1.9.20 Non-parametric Kruskal Wallis table for comparison of percent larval migration inhibition and worm strain (*H. contortus* susceptible, *H. contortus* resistant and *T. colubriformis* susceptible strain) for mimosa extract for the larval migration inhibition assay.

Test Statistics[a,b]

	percentmigration inhibition
Chi-Square	6.995
df	2
Asymp. Sig.	.030

a. Kruskal Wallis Test
b. Grouping Variable: Strain

Table A1.9.21 Non-parametric Kruskal Wallis table for comparison of percent exsheathed larvae for worm strain (*H. contortus* susceptible, *H. contortus* resistant and *T. colubriformis* susceptible strain) for calliandra condensed tannin for the larval exsheathment assay.

Test Statistics[a,b]

	Percentexsheathed
Chi-Square	11.185
df	2
Asymp. Sig.	.004

a. Kruskal Wallis Test
b. Grouping Variable: Strain

Table A1.9.22 Non-parametric Kruskal Wallis table for comparison of percent exsheathed larvae for calliandra condensed tannin concentration for the larval exsheathment assay.

Test Statistics[a,b]

	Percentexsheathed
Chi-Square	18.092
df	4
Asymp. Sig.	.001

a. Kruskal Wallis Test
b. Grouping Variable: CTconc

Table A1.9.23. Non-parametric Kruskal Wallis table for comparison of percent exsheathed larvae for time of exsheathment for calliandra condensed tannin for the larval exsheathment assay.

Test Statistics[a,b]

	Percentexsheathed
Chi-Square	128.402
df	3
Asymp. Sig.	< .001

a. Kruskal Wallis Test
b. Grouping Variable: time

Table A1.9.24 Non-parametric Kruskal Wallis table for comparison of percent exsheathed larva for calliandra condensed tannin concentration for *H. contortus* susceptible, *H. contortus* resistant and *T. colubriformis* susceptible strain for the larval exsheathment assay.

Test Statistics[a,b]

	Hs	Hr	Ts
Chi-Square	5.310	8.401	6.707
df	4	4	4
Asymp. Sig.	.257	.078	.152

a. Kruskal Wallis Test
b. Grouping Variable: CTconc

Table A1.9.25 Non-parametric Kruskal Wallis table for comparison of percent exsheathed larva for time of exsheathment for *H. contortus* susceptible, *H. contortus* resistant and *T. colubriformis* susceptible strain for calliandra condensed tannin for the larval exsheathment assay.

Test Statistics[a,b]

	Hs	Hr	Ts
Chi-Square	47.370	45.789	43.743
df	3	3	3
Asymp. Sig.	< .001	< .001	< .001

a. Kruskal Wallis Test
b. Grouping Variable: time

Table A1.9.26. Non-parametric Kruskal Wallis table for comparison of percent exsheathed larva for calliandra condensed tannin concentration for *H. contortus* susceptible strain for calliandra condensed tannin for the larval exsheathment assay.

Test Statistics[a,b]

	ten	twenty	forty	sixty
Chi-Square	11.067	13.033	12.133	6.710
df	4	4	4	4
Asymp. Sig.	.026	.011	.016	.152

a. Kruskal Wallis Test
b. Grouping Variable: CTconc

Table A1.9.27 Non-parametric Kruskal Wallis table for comparison of percent exsheathed larva for calliandra condensed tannin concentration for *H. contortus* resistant strain for calliandra condensed tannin for the larval exsheathment assay.

Test Statistics[a,b]

	ten	twenty	forty	sixty
Chi-Square	8.830	11.529	13.033	13.182
df	4	4	4	4
Asymp. Sig.	.065	.021	.011	.010

a. Kruskal Wallis Test
b. Grouping Variable: CTconc

Table A1.9.28 Non-parametric Kruskal Wallis table for comparison of percent exsheathed larva for calliandra condensed tannin concentration for *T. colubriformis* susceptible strain for calliandra condensed tannin for the larval exsheathment assay.

Test Statistics[a,b]

	ten	twenty	forty	sixty
Chi-Square	12.567	11.233	13.745	1.556
df	4	4	4	4
Asymp. Sig.	.014	.024	.008	.817

a. Kruskal Wallis Test
b. Grouping Variable: CTconc

Table A1.9.29 Non-parametric Kruskal Wallis table for comparison of percent exsheathed larvae for worm strain (*H. contortus* susceptible, *H. contortus* resistant and *T. colubriformis* susceptible strain) for mimosa extract for the larval exsheathment assay.

Test Statistics[a,b]

	Percentexsheathed
Chi-Square	6.641
df	2
Asymp. Sig.	.036

a. Kruskal Wallis Test
b. Grouping Variable: Strain

Table A1.9.30 Non-parametric Kruskal Wallis table for comparison of percent exsheathed larvae for mimosa extract concentration for the larval exsheathment assay.

Test Statistics[a,b]

	Percentexsheathed
Chi-Square	1.178
df	4
Asymp. Sig.	.882

a. Kruskal Wallis Test
b. Grouping Variable: CTconc

Table A1.9.31 Non-parametric Kruskal Wallis table for comparison of percent exsheathed larvae for time of exsheathment for mimosa extract for the larval exsheathment assay.

Test Statistics[a,b]

	Percentexsheathed
Chi-Square	146.503
df	3
Asymp. Sig.	< .001

a. Kruskal Wallis Test
b. Grouping Variable: time

Table A1.9.32 Non-parametric Kruskal Wallis table for comparison of percent exsheathed larva for mimosa extract concentration for *H. contortus* susceptible, *H. contortus* resistant and *T. colubriformis* susceptible strain for the larval exsheathment assay.

Test Statistics[a,b]

	Hs	Hr	Ts
Chi-Square	.730	.590	1.408
df	4	4	4
Asymp. Sig.	.948	.964	.843

a. Kruskal Wallis Test
b. Grouping Variable: CTconc

Table A1.9.33 Non-parametric Kruskal Wallis table for comparison of percent exsheathed larva for time of exsheathment for *H. contortus* susceptible, *H. contortus* resistant and *T. colubriformis* susceptible strain for mimosa extract for the larval exsheathment assay.

Test Statistics[a,b]

	Hs	Hr	Ts
Chi-Square	53.366	51.243	47.459
df	3	3	3
Asymp. Sig.	< .001	< .001	< .001

a. Kruskal Wallis Test
b. Grouping Variable: time

APPENDIX 2: SUMMARY OF *IN VIVO* AND *IN VITRO* ANTHELMINTIC EFFECTS OF GASTROINTESTINAL NEMATODES

Table A2.1 Summary if *in vivo* experiments on anthelmintic effects of gastrointestinal nematodes (NE: no effect).

Plant of Origin	Treatment and period	Animal	Tannin	Abomasal sp.	Intestinal sp.	Worm Burden	Fecundity	Female:Male	FEC	LWG compared to control	Reference
sericea lespedeza	75% of intake- 49 days	goats	22.4%	*H. contortus*		NE		NE	↓		Armour et al. (1966)
sericea lespedeza	75% of intake-5 weeks	goats	22.4%	*H. contortus* *T. circumcincta*	*T. colubriformis*	↓ ↓ ↓		↓females ↓females ↓females	↓ ↓ ↓		Fox (1997)
sericea lespedeza (pelleted)	75% of intake-4 weeks	goats	~6.5%	*H. contortus* *O. circumcincta*		↓ (75%) NE			↓ (70%)		Roseby (1977)
Acacia mearnsii	18g containing 18% CT/animal /week-84 days	sheep	18%	*H. contortus*	*T. colubriformis* *Cooperia sp.* *O.columbianum* *S.papillosus* *Trichuris globulosa* *Moniezia expansa*	↓ ↓ NE NE NE NE		↓males	↓ (all species)		Simpson et al. (1997)
C. calothyrsus	0.7kg – 1 wk	lambs	CT	*H. contortus*	*T. colubriformis*	NE		NE	-	-	Parker and Palmer (1991)
L. pedunculatus	28 days	lambs	CT	*O. circumcincta*	*T. colubriformis*				↓	higher	(Niezen et al. 1993)
sulla	28 days	lambs	CT	*O. circumcincta*	*T. colubriformis*				NE	NE	Niezen et al. (1993)
sulla	4kg/hd/d- 6 wks	lambs	CT	*O. circumcincta*	*T. colubriformis*				↓ NE		Niezen et al. (1994)
sulla	28 days	lambs	CT	*O. circumcincta* *T.axei*	*T. colubriformis*				NE ↓ ↓	higher	Niezen et al. (1995)

Table A2.1 Continued

Plant of Origin	Treatment and period	Animal	Tannin	Abomasal sp.	Intestinal sp.	Worm Burden	Fecundity	Female:Male	FEC	LWG compared to control	Reference
L. pedunculatus *L. corniculatus* *sulla*		lambs	CT	√	√	NE NE ↓ (abomasal & intestinal)			NE NE ↓ (40%)	higher higher	Robertson et al. (1995)
L. pedunculatus	28 days	lambs	CT	*O. circumcincta*	*T. colubriformis*	↓ NE		↓ females NE	↓ NE	higher	Niezen et al. (1998b)
L. pedunculatus *L. corniculatus* *sulla*	Ad lib (>3kg/hd/d)	lambs	8% 4% 12%	*Haemonchus* *Ostertagia* *Trichostrongylus*	*Trichostrongylus* *Cooperia* *Nematodirus*	NE NE ↓ (all species)			NE NE ↓	higher higher	Niezen et al. (1998a)
Quebracho extract	30 & 60g extract/kg fresh food Drenched at 8 % w/w of intake– 1 week	lambs	73%		*T. colubriformis*				↓		Athanasiadou et al. (2000)

Table A2.1 continued

Plant of Origin	Treatment and period	Animal	Tannin	Abomasal sp.	Intestinal sp.	Worm Burden	Fecundity	Female:Male	FEC	LWG compared to control	Reference
Quebracho extract	Drenched as % w/w of intake– 3 days. 8% 8% 4%, 8% & 16% 4%, 8% & 16%	lambs	73%	*H. contortus* *T. circumcincta*	*T. colubriformis* *N. battus*	NE NE ↓ (8% only) ↓ (8% only)	NE NE ↓ (16% only) ↓ (16% only)	NE NE NE NE	NE NE ↓ ↓		Athanasiadou et al. (2001)
Cassava Hay	1kg/hd/d-4 weeks	Buffalo/ Cattle	3.3%	GI Nematodes					↓		Netpana et al. (2001)
sulla	5kg/d DM	lambs	CT	*O. circumcincta*	*T. colubriformis*	↓ NE			↓ NE		Niezen et al. (2002)
Acacia nilotica	40% DM- 8 weeks	goat	Mostly HT	*H. contortus*	-	↓ 10%			↓	Lower	Kahiya et al. (2003)
Acacia karoo	40% DM- 8 weeks	goat	CT	*H. contortus*	-	↓ 34%			↓	Lower	Kahiya et al. (2003)
C. calothyrsus	100% DM-2 weeks	lambs	CT	*H. contortus*	*T. colubriformis*				↓		Cresswell (2007)
sericea lespedeza (leaf meal pellets)	75% 28 days	goats	CT	*H. contortus*					↓		Kommuru et al. (2015)
Sainfoin	Fed for 42 days	cattle	CT	*O. ostertagia*	*Cooperia onocophora*				↓ NE		Desrues et al. (2015)

Table A2.2 Summary of findings from *in vitro* experiments on anthelmintic effects of gastrointestinal nematodes.

Plant of Origin	Treatment	Animal	Abomasal sp.	Intestinal sp.	EHA	LDA	LMIA (%)	LEA	LFIA	Reference
Lotus pedunculatus *Lotus corniculatus* *Onobrychus viciifloia* *Hedysarum coronarium (sulla)*	25-900 µg/ml CT	Sheep		*T. colubriformis*	39%	↓ with ↑ [CT] ↓ with ↑ [CT] ↓ with ↑ [CT] ↓ with ↑ [CT]	21-39			Molan et al. (1999)
Lotus pedunculatus *Lotus corniculatus* *Onobrychus viciifloia* *Hedysarum coronarium (sulla)*	1200 µg/mL	Deer (L_3)	GI nematodes (Mostly Ostertagia-type sp.)				**EX** **EN** 68 72 55 51 77 75 57 63			Molan et al. (2000b)
Hedysarum coronarium (sulla)	50-1000 mg/mL	Sheep (L_3)	O. *circumcincta* *H. contortus*	*T. colubriformis*			**RF** **AF** 37 26 59 79 72 81			Molan et al. (2000a)
Quebracho extract	1.25% – 10% PA 0.75% – 12% PA 1.25% – 10% PA	Sheep	*H. contortus* *T. circumcincta*	*T. vitrinis*	No effect No effect No effect	↓ with ↑ [CT] ↓ with ↑ [CT] ↓ with ↑ [CT]				Athanasiadou et al. (2001)
Lotus pedunculatus *Lotus corniculatus* *Dorycnium pentaphyllum* *Dorycnium rectum* *Rumex obtusifolius*	50-900 µg/mL	Sheep	*T. circumcincta*		↓ with ↑ [CT] ↓ with ↑ [CT] ↓ with ↑ [CT] ↓ with ↑ [CT] ↓ with ↑ [CT]					Molan et al. (2002)

Table A2.2 Continued

Plant of Origin	Treatment	Animal	Abomasal sp.	Intestinal sp.	EHA	LDA	LMIA (%)	LEA	LFIA (%)	Reference
Onobrychis viciifolia *Rubus fructicosus* *Quercus robur* *Corylus avellana* *Onobrychis viciifolia* *Rubus fructicosus* *Quercus robur* *Corylus avellana* *Onobrychis viciifolia* *Rubus fructicosus* *Quercus robur* *Corylus avellana*	300-1200 µg/mL	Goats	*H. contortus* *T. circumcincta*	*T. colubriformis*			reduced NE reduced reduced NE reduced reduced reduced reduced reduced NE NE			Paolini et al. (2004)
Sarthamnus scoparius *Pinus sylvestris* *Erica reigena* *Castanea sativa*	600ug/mL	Goats	*H. contortus*	*T. colubriformis*				NE delayed delayed 100% inhibition		Bahuaud et al. (2006)
C. calothyrsus		Sheep	*H. contortus*	*T. colubriformis*	reduction					Cresswell (2007)
Lisyloma latisiliquum *Leacana leucocephala* *Acacia pennatula* *Piscidia piscipula*	1200 µg/mL (after 70 min)	Goats		*T. colubriformis*				4% 3.7% 0% 11.4%		Alsonso-Diaz et al. (2008a)
Lisyloma latisiliquum *Leacana leucocephala* *Acacia pennatula* *Piscidia piscipula*	1200 µg/mL (after 60 min)	Goats	*H. contortus*					5.1% 10.64% 2.78% 4.85%		Alsonso-Diaz et al. (2008b)
Manihot esculenta	Leaf extracts (150-2400 µg/mL)	Sheep	*H. contortus*		No effect	↓	NE			Marie-Magdeleine et al. (2010)

Table A2.2 continued

Plant of Origin	Treatment	Animal	Abomasal sp.	Intestinal sp.	EHA	LDA	LMIA (%)	LEA	LFIA (%)	
Pistacia lentiscus *Pyrus spinosa* *Quercus coccifera* *Ceratonia silique* *Olea europaea* *Castanea sativa* *Ceratonia silique* *Onobrychis viciifolia*	150-1200 µg/mL	Sheep	*H. contortus*				reduced reduced reduced reduced NE reduced reduced reduced			Manolaraki et al. (2010)
Lotus pedunculatus *Lotus corniculatus* *Dorycnium pentaphyllum* *Dorycnium rectum* *Rumex obtusifolius*	900 µg/mL	Sheep	*T. circumcincta*		53% 68% 51% 60% 46%	↓ with ↑ [CT] ↓ with ↑ [CT] ↓ with ↑ [CT] ↓ with ↑ [CT] ↓ with ↑ [CT]				Molan and Faraj (2010)
Lotus pedunculatus *Lotus corniculatus* *Onobrychis viciifolia*	160ug/mL	Calves	*O. ostertagia*	*C. oncophora*				delayed	> 95% 100	Novobilsky et al. (2011)
Viscum rotubdufolium *Viscum verrucosum* *Tapinanthus oleifolus* *Grewia flava*	EH: 500 µg/mL LD:1/mL LMI: 500 µg/mL	Sheep	*H. contortus* (LMIA, EHA, LDA) *T. circumcincta* (EHA, LDA)	*T. colubriformis* (EHA, LDA)		Variable results	0 0 14.6 17.2			Tibe et al. (2013)
Hedysarum coronarium (fresh/hay)	150-1200 µg/mL	Goats	*H. contortus*					Delayed Fresh>hay		Aissa et al. 2015
Willow, Talia flowers, black and red current leaves	Dose response	Sheep/goat	*H. contortus*					inhibited		Klongsiriwet et al. (2015)

NE: No effect; EN: ensheathed larvae; EX: exsheathed larvae; RF: incubated in rumen fluid; AF: incubated in abomasal fluid; EHA: egg hatch; LDA: larval development; LMIA: larval migration; LEA: larval exsheathment; LFIA: Larval feeding inhibition